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BIOCHEMICAL ASPECTS OF CHROMOSOME BREAKAGE

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I. Introduction

In most cytological studies on the effects of chromosome-breaking agents in plants, the experimental material has been either microspores of the spiderwort, *Tradescantia paludosa* ($n = 6$), or root tips of the broad bean, *Vicia faba* ($2n = 12$). Thus, the effects are studied in haploid cells in *Tradescantia* and in diploid cells in *Vicia*.

Both these materials have their advantages and disadvantages. In *Tradescantia*, the developmental stage of the cells at the time of treatment can be determined rather accurately due to synchronized division of cells in an individual anther, but the material is less suitable for treatments with solutions of radiomimetic chemicals. The treatment of root tips with agents dissolved in water provides no difficulties, but the mitotic stage at the time of treatment can be determined satisfactorily only in cases where the cells have been "marked" before the treatment (Revell, 1953; Kihlman, 1955a; Evans *et al.*, 1957).

The six chromosomes in a *Tradescantia* microspore have a rather similar appearance. The chromosome complement in the root tips of *Vicia faba* consists of five pairs of chromosomes with subterminal centromeres (S-chromosomes) and one pair with median centromeres (M-

chromosomes). In an M-chromosome, a large satellite is separated from the rest of the chromosome by a nucleolar constriction. An M-chromosome is about twice as long as an S-chromosome, the ratio of the total metaphase lengths of the M-chromosomes and the S-chromosomes being 1:2.2 (Revell, 1953). Thus, if breaks occurred at random, the ratio of the total number of breaks in M-chromosomes and in S-chromosomes would be 1:2.2. After treatments with radiomimetic chemicals, the resulting ratios usually diverge greatly from 1:2.2, indicating that breakage is nonrandom.

Other differences exist between the two experimental materials in addition to those already mentioned. The requirements of the problem to be studied would necessarily determine the investigator's choice of the two.

In the past, studies on chromosome breakage were performed mainly from a biophysical point of view (see, e.g., Lea, 1946), when they could not be classified as descriptive cytology. During the last few years, however, the biochemical aspects of chromosome breakage have come to the front. It has been realized that the chromosome is a dynamic rather than a static system, and that biochemical processes are involved in the formation of structural chromosomal changes. Of those biochemical processes, oxidative phosphorylation seems to be one of the most significant.

In the present review article, the main theme has been the effects of oxygen, respiration, and oxidative phosphorylation on the production of chromosomal aberrations by radiations and chemicals. Accordingly, of the known cases of agents with chromosome-breaking effects, only those in which the influence of oxygen has been studied have been included.

II. Oxygen, Respiration, and the Induction of Chromosomal Aberrations

A. Oxygen-Dependent Chromosome Damage

1. *X-rays*

a. *Effect of Oxygen on Breakage.* The significance of oxygen concentration in the chromosome-breaking effects of X-rays appears in studies performed by Thoday and Read (1947) with root tips of *Vicia faba* as experimental material. They found that the frequencies of chromosomal aberrations produced by a given X-ray dosage in nitrogen were about one-third of those produced by the same dosage in oxygen. The fact that the replacement of nitrogen by nitrous oxide, carbon dioxide, or hydrogen had no influence on the effect indicated that it

was the lack of oxygen and not the presence of nitrogen which was responsible for the reduction of the frequencies of X-ray-induced chromosomal aberrations.

The results of Thoday and Read were soon confirmed by a series of investigations performed by Giles and his collaborators with microspores of *Tradescantia paludosa* as the experimental material. The aberrations were studied in the first microspore mitosis. When the irradiations were performed in air or in oxygen, the frequencies of both chromatid (Riley *et al.*, 1952) and chromosome aberrations (Giles and Riley, 1949; Giles and Beatty, 1950) were considerably higher than those obtained when the same X-ray dosage was given in nitrogen, argon, or helium. It was not possible, however, to suppress the X-ray effect completely by removing the oxygen. Even under completely anaerobic conditions, the effect obtained was considerable. The studies by Giles and his collaborators further showed that it was the oxygen concentration during the treatment itself which was significant in the effect; pre- and post-treatments with various oxygen concentrations were without influence on the frequencies of chromosomal aberrations (Giles and Riley, 1950). The effect of oxygen was an immediate one, which appeared from the fact that it was possible to affect the aberration frequency by altering the oxygen concentration during the treatment.

Giles and his collaborators also made attempts to determine in more detail the influence of oxygen concentration on the X-ray effect. As appears from Fig. 1, the experiments showed that the frequency of aberrations increased rapidly when the oxygen concentration in the gas phase was increased from 0 to 10%. Above this level the increase became gradually less marked, being insignificant between 21 and 100% oxygen (Giles and Riley, 1950; Giles and Beatty, 1950; Riley *et al.*, 1952). Since similar relations between oxygen concentration and X-ray effect were obtained using other experimental materials and methods, it seemed as if this relationship was characteristic of the X-ray effect.

Recently, Alper and Howard-Flanders (Alper and Howard-Flanders, 1956; Howard-Flanders and Alper, 1957) found that the lethal effect of X-rays on microorganisms was enhanced by such low concentrations of oxygen as 0.07%. The maximum enhancing effect was reached when the oxygen concentration in the gas phase was about 2.5%. In the experiments of Alper and Howard-Flanders, the gas mixture was passed through the treatment vessel both for a period before and during irradiation. The gas mixture was introduced into the vessel through a sintered glass filter so that it passed through the solution as a vigorous stream of fine bubbles. By this method, a rapid stirring and a large area of interface between liquid and gas was ensured.

Alper and Howard-Flanders (1956) found that the sensitivity of microorganisms to X-rays at different oxygen concentrations could be fitted by the equation:

$$\frac{S}{S_N} = \frac{m[O_2] + K}{[O_2] + K}$$

where S is the sensitivity at the oxygen concentration $[O_2]$ and S_N the sensitivity in the absence of oxygen. The factor m represents the maximum enhancement ratio, or the ratio between a given dose and that dose which in oxygen produces the same effect as the given dose in the absence of oxygen. K is that oxygen concentration in μM which gives

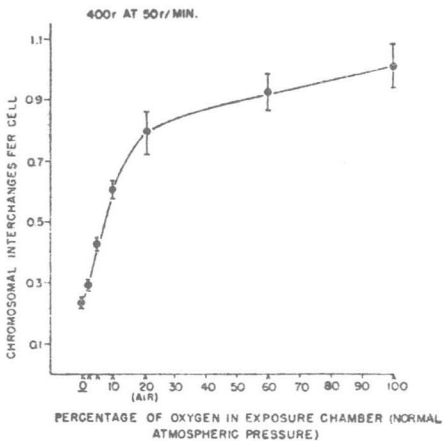

FIG. 1. Relation between percentage of oxygen and yield of X-ray-induced chromosome interchanges in *Tradescantia* microspores. (From Giles and Beatty, 1950.)

a sensitivity of $(m + 1)/2$ times the radiosensitivity in nitrogen. When $m = 3$, as is usually the case when X-rays are used, the sensitivity is increased by the oxygen concentration K to a level halfway between the maximum sensitivity and the nitrogen sensitivity.

In the experiments of Howard-Flanders and Alper (1957), an m-value of 2.92 and a K-value of 4.0 ± 0.4 μM were obtained for the bacterium *Shigella flexneri*. Values of the same order of magnitude were obtained by Howard-Flanders and Alper in experiments with haploid yeast, *Saccharomyces cerevisiae*. The K value for *Shigella flexneri* has subsequently been corrected by Howard-Flanders to 1 μM, however (Howard-Flanders, 1958; Howard-Flanders and Jockey, 1960).

In a study of the influence of oxygen concentration on the frequencies of X-ray-induced abnormal anaphases in mouse ascites tumors, Desch-

ner and Gray (1959) obtained a K value of $5 \pm 2\ \mu M$. Dewey (1960) found $K = 8.5\ \mu M$ for human cells in tissue culture. The K values of Deschner and Gray, and of Dewey are of the same order of magnitude as those obtained by Howard-Flanders and Alper. In all these cases, where the experimental material consisted of suspensions of single cells, oxygen proved to be active in concentrations about thirty times lower than those effective in the experiments of Giles and collaborators. According to Howard-Flanders and Alper (1957), this difference is probably due to the fact that when the experimental material consists of organized tissues, the available oxygen is to a large extent consumed in the outermost cell layers as a result of their respiratory activity. Therefore, at low oxygen concentrations anaerobic conditions are likely to prevail in the central parts of the tissue.

This suggestion of Howard-Flanders and Alper was supported by results obtained by Kihlman (1958b, 1959c, 1961c) in studies on the influence of respiratory inhibitors on the frequencies of chromosomal aberrations produced at different oxygen concentrations by a given X-ray dosage in the root tips of *Vicia faba*. In most experiments cupferron (*N*-nitrosophenylhydroxylamine—ammonium salt) was used as the respiratory inhibitor. Cupferron, a chelating agent, was found to inhibit bean root respiration effectively at pH values between 5 and 6. Some of the results obtained in these studies appear in Fig. 7A.

It was found that in the presence of cupferron an increase of the X-ray effect was obtained at oxygen concentrations as low as 0.1% (corresponding to 1.3 μM in solution at 22°C.). As a rule, the maximum effect was obtained when the oxygen concentration in the gas phase reached 2% (26 μM in solution). In experiments performed in the absence of cupferron, oxygen concentrations below 5% did not produce a significant increase in the frequencies of chromosomal aberrations. A very marked increase was obtained in these experiments at oxygen concentrations between 5 and 21%, and the maximum effect was obtained when the gas phase contained about 50% oxygen. A K value of $4.2 \pm 0.9\ \mu M$ was obtained in the presence of cupferron as compared to about 130 μM in the absence of the inhibitor.

The results indicate that when respiration is inhibited, the same relationship exists between oxygen concentration and X-ray effect in experiments with organized tissues as in experiments with suspensions of single cells. Apparently, oxygen is able to diffuse freely into the central parts of the tissue only when respiration is inhibited.

In addition to cupferron, the respiratory inhibitors azide, cyanide, and carbon monoxide were tested (Kihlman, 1959c, 1961c). Most of the experiments were performed in the presence of 1% oxygen in the gas

phase (about 14 μM in solution at 20°C.). This oxygen concentration, in the absence of respiratory inhibitors, had no effect on the radiosensitivity of bean roots. When respiration was inhibited by azide (at pH 5), cyanide, or carbon monoxide, the frequency of chromosomal aberrations produced by a given dosage of X-rays was of approximately the same order of magnitude in the presence of 1% oxygen as in air.

If the effect of respiratory inhibitors on X-ray sensitivity is to abolish the oxygen gradient existing in organized tissues, they should be effective only in the presence of oxygen. This appears to be the case for all the inhibitors tested (Kihlman, 1959c, 1961c). Furthermore, they should be effective only at oxygen concentrations below 21%, since at higher oxygen concentrations the gradient should have no influence on X-ray sensitivity. However, as appears from Fig. 7A, the maximum effect in the presence of cupferron is considerably higher than that obtained in the absence of the inhibitor. No satisfactory explanation has as yet been obtained for this increase in maximum effect, but apparently it has nothing to do with the oxygen effect.

Recent experiments have shown that cupferron treatments are effective only when given before and during the irradiation (Kihlman, 1961c). Post-treatments with this inhibitor have no effect. Similar results were obtained when carbon monoxide was used as a respiratory inhibitor (Kihlman, 1961c). These results are entirely in agreement with the explanation given above for the effect of respiratory inhibitors on X-ray sensitivity.

Respiratory inhibitors such as azide, cupferron, and cyanide are convenient to use when the experimental material is immersed in an aqueous solution during irradiation, as in the case of bean roots. The difficulties are considerably greater when *Tradescantia* microspores are the experimental material. Of the inhibitors discussed above, only carbon monoxide can be conveniently and effectively applied in this case.

Evans and Neary (1959) have shown, however, that it is possible to do without the inhibitors in *Tradescantia* if isolated germinating pollen tubes are used as experimental material. In their experiments the irradiations were performed 4 hours after the pollen had been sown on slides coated with a lactose-agar medium containing 0.01% colchicine. At this time, the generative nucleus of the pollen grain had passed into the pollen tube. In this material, the oxygen concentration cannot be markedly reduced by respiration and, consequently, no oxygen gradient exists.

Evans and Neary determined the X-ray sensitivity at different oxygen concentrations and obtained a curve (Fig. 2), which is considerably steeper than that obtained by Giles and Beatty (Fig. 1). The K value

found by Evans and Neary was 10.2 ± 2.8 μM. According to the calculations of Alper (1956), the curve published by Giles and Beatty (1950) corresponds to a K value of 135 μM.

How then is the striking ability of low concentrations of oxygen to enhance X-ray sensitivity to be explained?

A reasonable explanation seemed to be that oxygen influences X-ray sensitivity by means of the respiratory chain, i.e., that the oxygen effect is tied up with oxidative metabolism. Actually, such an explanation

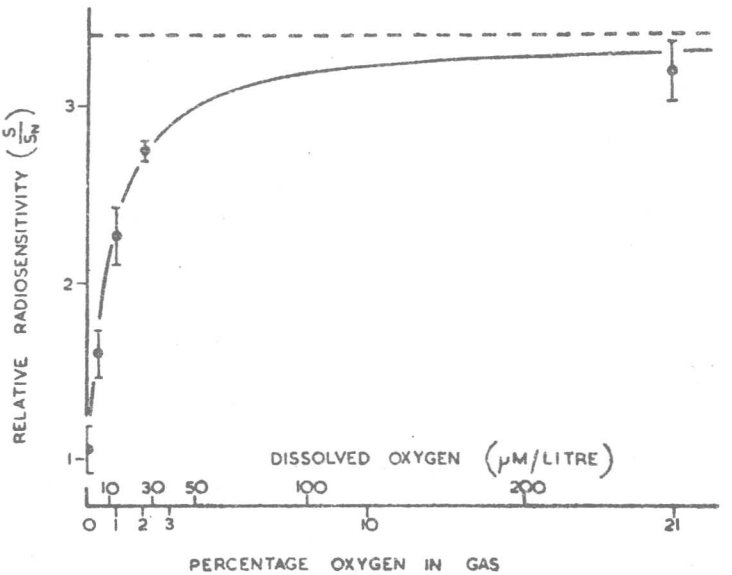

FIG. 2. Relation between percentage of oxygen and the relative yield of X-ray-induced isochromatid aberrations in *Tradescantia* pollen tubes. (From Evans and Neary, 1959.)

appears to be valid for the oxygen effects observed in connection with the production of chromosomal aberrations by certain radiomimetic chemicals (Kihlman, 1955c, 1956; Rieger and Michaelis, 1960a). In the cases of the radiomimetic chemicals referred to, the chromosome-breaking effect was inhibited to the same extent by respiratory inhibitors as by anoxia. However, the X-ray effect on chromosome structure is not suppressed by respiratory inhibitors (Kihlman, 1955c, 1959c), nor is it inhibited by agents such as 2,4-dinitrophenol, which uncouple phosphorylation from respiration (Merz, personal communication; Kihlman, unpublished). Thus, it must be concluded that, in the case of X-rays, oxidative metabolism is not involved in the oxygen effect.

Thoday and Read (1949) found that the production of chromosomal aberrations by alpha rays in root tips of *Vicia faba* was influenced very slightly by oxygen tension. At that time it was known that irradiation of water with X-rays resulted in the production of hydrogen peroxide (H_2O_2) only if the water contained dissolved oxygen. In the case of alpha rays, on the other hand, hydrogen peroxide was formed regardless of whether the water contained dissolved oxygen or not. Thus, there appears to be a striking correlation between the influence of oxygen on the production of chromosomal aberration by ionizing radiations, on the one hand, and on the production of hydrogen peroxide by the same radiations, on the other. Both phenomena are independent of the oxygen concentration in the case of alpha rays and enhanced by oxygen in the case of X-rays. Therefore, Thoday and Read (1949) concluded that hydrogen peroxide "has some influence on the processes involved in chromosome structural change." The fact that hydrogen peroxide proved to be mutagenic in *Neurospora* (Wagner *et al.*, 1950), rendered the hypothesis still more attractive.

Another species considered to be involved in chromosome structural damage is the hydroperoxyl radical (HO_2), which also is formed when water is irradiated with X-rays in the presence of oxygen (e.g., Gray, 1953). During the last few years, however, evidence has accumulated against the idea that radiation exerts its effect in biological systems through H_2O_2 or HO_2-radicals formed in the water. This evidence has been thoroughly summarized and discussed by Alper (1956).

A fact not easily compatible with the "water radical" hypothesis as an explanation for the oxygen effect is the discovery by Howard-Flanders (1957) that nitric oxide (NO) enhances the lethal effect of X-rays on bacteria to the same extent as oxygen. Subsequently, Howard-Flanders also studied the enhancing influence of NO on the radiation sensitivity of yeast (Howard-Flanders and Jockey, 1960). He points out (Howard-Flanders, 1958; Howard-Flanders and Jockey, 1960) that NO reproduces the influence of oxygen on the radiation effect both qualitatively and quantitatively.

These results have been confirmed by Gray *et al.* (1958), Kihlman (1958a, 1959d), and Dewey (1960), using mouse ascites tumor cells, *Vicia* root tips, and human cells, respectively, as experimental materials.

In dry systems NO seems to have the opposite effect. Powers *et al.* (1959, 1960) found that NO protected dry spores of *Bacillus megaterium* against X-ray damage, and a similar effect of NO was found by Sparrman *et al.* (1959) for dry seeds of *Agrostis stolonifera*.

Howard-Flanders (1958, 1959) has also provided a possible explanation for the oxygen and nitric oxide enhancement of X-ray damage, and

for the fact that the effect of alpha radiation is independent of oxygen concentration. According to this hypothesis, two types of primary changes are induced by ionizing radiation in those molecules of the cell which are essential for multiplication. One of the two types results in a permanent and lethal damage only in the presence of oxygen (or nitric oxide), whereas the other results in a lethal effect regardless of whether oxygen is present or not. In principle, the oxygen-dependent change may be produced when the number of ions formed (i) along a segment of length t of the track of the ionizing particle, fulfills the condition $r \leqslant i < n$, while an oxygen-independent injury may be produced when $i \geqslant n$. Which of the two types will predominate is, therefore, dependent on the ion density of the radiation. Alpha radiation is a densely ionizing radiation, and, therefore, the relative probability that a change of the oxygen independent type will occur is high.

The reasons that n or more ionizations are needed for the production of an oxygen-independent change may be the following:

Apparently, the result of the primary change, which Howard-Flanders assumes to be a carbon radical, has a very short lifetime so that the original configuration of a radiation-affected molecule in the cell is usually restored within a fraction of a second. A prerequisite for permanent damage to arise is the reaction of the radiation-induced radical with another radical. The latter may be radiation-induced, such as an OH-radical produced in water, or it may be stable, such as oxygen or nitric oxide. When the radiation is sparsely ionizing, the distance between the radicals formed is usually too great for a reaction between them to occur. Therefore, the effect is dependent in this case on the concentration of oxygen (or nitric oxide) molecules in the cell. In the case of alpha rays, on the other hand, the radicals are formed closely enough for reactions between them to occur, and the effect becomes more or less independent of oxygen concentration. Oxygen and nitric oxide owe their radical properties and ability to react with carbon radicals to the fact that they possess unpaired electrons in π molecular orbitals.

b. *Effect of Oxygen on Rejoining.* It was stated above that the effect of oxygen on the production of chromosomal aberrations by X-rays is not tied up with the oxidative metabolism of the cell (see page 7). However, Wolff and Luippold (1955) have shown that, under certain conditions, the frequencies of chromosomal aberrations produced by a given dose of X-rays are influenced by oxidative metabolism. Such an influence is observed when the dosage is given in fractions or when the dose rate is low.

The fundamental studies by Sax (1939, 1940, 1941) demonstrate that

the frequency of exchanges produced by a given X-ray dosage is dependent on the dose rate. When the duration of the irradiation period is prolonged beyond a certain limit, the frequency of exchanges decreases. The time of irradiation necessary to produce this decrease appears to be different in different organisms, types of cells, and division stages. Studies by Sax (1939, 1941) and Fabergé (1940) have shown that a similar decrease of the X-ray-induced exchanges can be obtained by fractionating the dose.

The decrease in frequency of exchanges obtained when the dose is fractionated or the dose rate decreased may be explained on the basis of the breakage-first hypothesis in the following way:

Exchanges can be formed between breaks that are open at the same time and close enough in space. Breaks remain open only for a relatively short time. When the dose rate is high, all the breaks produced are open at the same time and numerous exchanges occur. When the dose rate is low, on the other hand, the chances for exchanges to occur are less, since breaks produced at the beginning of the irradiation period have had time to rejoin before the irradiation is finished and are, therefore, unable to take part in exchanges with breaks produced toward the end of the irradiation period. The decrease obtained by dose fractionation is similarly explained as due to the rejoining of breaks produced by the first fraction of the dose before the second fraction is given. The fact that dose-rate and fractionation effects are found in the case of X-rays indicates that the two breaks involved in an exchange are, as a rule, formed by two separate ionizing particles at high dose rates. In the case of fast neutrons, the two breaks involved in an exchange appear to be induced by the same particle, since no dose-rate or fractionation effects have been observed.

Wolff and his collaborators (Wolff, 1954, 1960a; Wolff and Atwood, 1954; Wolff and Luippold, 1955, 1956a, 1956b) have performed detailed studies on the effects of dose rate and dose fractionation on the frequencies of "two-hit aberrations" in the root tips of *Vicia faba*. In these experiments, seeds were irradiated after having been soaked in water for 18 to 24 hours. The effect was studied in the first root tip mitosis. All the aberrations produced by this method were of the chromosome type.

The effect of the duration of irradiation on the number of "two-hit aberrations" is shown in Fig. 3. When the time of irradiation was prolonged from ½ to 1 minute, the number of aberrations was diminished by approximately one-third. This happened both in the presence and in the absence of oxygen. The frequency of "two-hit aberrations" was unaffected when the duration of exposure was prolonged from 1 to 120