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*THE EFFECTS OF CARBON MONOXIDE AND OXYGEN ON THE FREQUENCY OF X-RAY INDUCED CHROMOSOME ABERRATIONS IN TRADESCANTIA**

BY EDWARD D. KING, HOWARD A. SCHNEIDERMAN AND KARL SAX†

BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY

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Though the end results of shortwave radiation have been frequently studied and are well understood, little is known concerning the mechanism whereby radiant energy produces its effects. Nor is there specific information concerning the way in which the physiological state of the cell conditions its response to irradiation. The recent reviews of Catcheside,[1] Hayden and Smith,[2] Sax,[3] Sparrow,[4] and Sparrow and Rubin[5] discuss various agents which modify the genetic and cytological effects of x-rays. Among these agents oxygen appears of signal interest.

Experiments of Thoday and Read,[6] Hayden and Smith,[2] Giles and Riley,[7] Giles, Beatty and Riley,[8] Riley, Beatty and Giles,[9] and Conger and Fair-

child[10] have shown that the availability of oxygen to cells is an important factor in radiosensitivity. Under anaerobic conditions radiation-induced inhibition of growth in barley and increase in frequency of chromosomal aberrations (microspores of Tradescantia and root tips of *Vicia faba*) were much less pronounced than in air. Contrariwise, oxygen tensions above the normal 21% caused an increase in chromosomal aberrations in the microspores of Tradescantia—a result which suggests that respiratory metabolism in some way is correlated with the frequency of chromosome aberrations. In the present study this possibility has been examined by studying the relation of heavy-metal-containing respiratory enzymes to the response of Tradescantia microspores to irradiation. The possible role of flavoproteins is also appraised.

Materials and Methods.—Tradescantia paludosa Anderson and Woodson was used in all experiments. Cytological observations were made of chromosomal aberration types four to five days after irradiation. The principal types of aberrations analyzed at this period are "interchanges" (dicentrics and centric rings).

The experimental material was placed inside a cylindrical lucite chamber, $4^1/_2$ in. O.D. × 4 in. I.D. × 18 in. long, fitted with brass endplates, gaskets and needle valves. The inflorescences were inserted in an upright position through holes in a waxed cardboard container filled with spring water. The container was placed in the center of the lucite chamber to insure uniform exposure and minimize secondary radiation from the brass endplates. Dosage was measured with a dosimeter placed at the position of the inflorescences in a lucite chamber identical with the experimental chamber. This chamber was placed next to the experimental chamber. In all experiments the dosage rate and total dosage were constant, i.e., 40 r/min. for 10 minutes.

The gases used in the experiments were obtained in commercial cylinders and assayed as follows:

Nitrogen (Airco) 99.5% N_2 plus 0.5% O_2.

Oxygen (Airco) 99.5% O_2 plus 0.5% N_2.

CO (Matheson Co.) 96.8% CO, 0.36% CO_2, 0.97% H_2, 1% N_2, 0.8% saturated hydrocarbons, 1.19 mg. Fe/liter, 0.32 mg. S/liter.

Prior to its use the carbon monoxide was filtered through 10% NaOH solution to remove the CO_2. Total gas pressure was measured by gauges calibrated in psi.

The inflorescences were enclosed in the air-filled lucite chamber and then compressed to the desired pressure with the gas under consideration. When sealed, the chamber thus contained one atmosphere of air plus a positive pressure of the experimental gas. The use of positive pressures permits several peculiar advantages. First, there is no question as to whether the experimental gas reaches the microspores through the layer of

air enclosed by the sepals and petals of the buds. Moreover, since at least 20.9% of an atmosphere of oxygen is present (the amount present normally in air), anoxia can play no role. The average time of compression was four minutes and the timing of exposure to the gas was calculated from the moment the final pressure was attained. The sealed chamber containing inflorescences was kept at room temperature for from 15 minutes to four hours, depending on the experiment, and then placed in the x-ray machine alongside the chamber containing the dosimeter. Irradiation was then performed at room temperature. In each experiment this

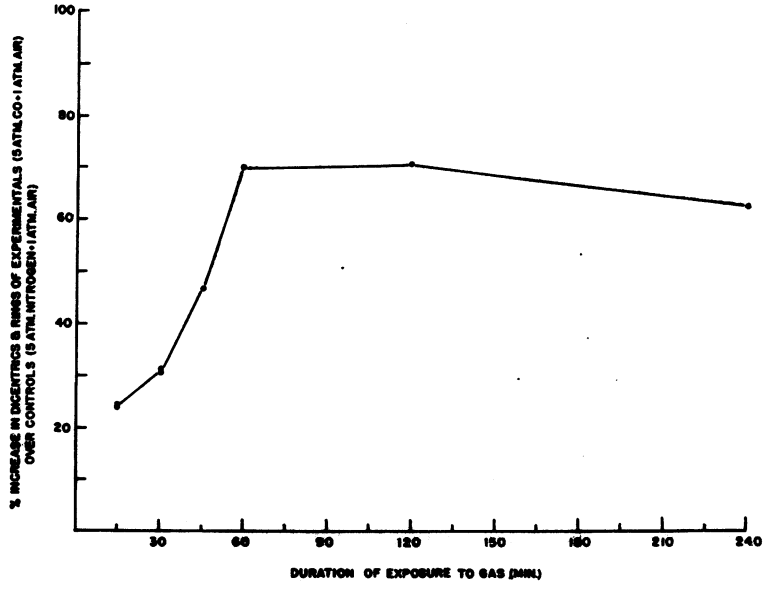

FIGURE 1

Curve showing the effect of time of exposure to carbon monoxide and to nitrogen on dicentrics and centric rings induced in Tradescantia microspores by x-rays.

procedure was carried out twice—once with the experimental gas (usually CO) and once with the control gas (usually N_2). The two experimental chambers were compressed and later irradiated within 30 minutes of each other. After exposure the chambers were decompressed slowly for 30 minutes and then opened to air.

In general, from 10 to 15 inflorescences were exposed in each chamber and as many slides as possible made four to five days following irradiation.

Results and Discussion.—The experimental results are presented in table 1 and shown graphically in figure 1. All the scoring was done by one investigator (E. K.). The first column in table 1 indicates the composition

TABLE 1

FREQUENCIES OF DICENTRICS AND CENTRIC RINGS INDUCED IN TRADESCANTIA MICROSPORES EXPOSED TO X-RAYS IN MIXTURES OF AIR, NITROGEN, OXYGEN AND CARBON MONOXIDE. ALL DOSAGES ADMINISTERED AT A CONSTANT INTENSITY OF 40 R/MIN. FOR 10 MINUTES

(For further discussion, see text)

EXPERIMENTAL				CONTROL			
GAS MIXTURE	DURATION OF EXPOSURE TO gas, min.	CHROMOSOMES COUNTED	PER CENT DICENTRICS AND RINGS	GAS MIXTURE	CHROMOSOMES COUNTED	PER CENT DICENTRICS AND RINGS	PER CENT INCREASE OF DICENTRICS AND RINGS OF EXPERIMENTALS OVER CONTROLS
5 atm. CO + 1 atm. air[a]	60	3000	0.0	5 atm. N_2 + 1 atm. air[a]	3000	0.0	0.0
5 atm. CO + 1 atm. air	60	4200	9.2	5 atm. N_2 + 1 atm. air	3000	9.1	Negligible
5 atm. CO + 1 atm. air	15	1542	11.3	5 atm. N_2 + 1 atm. air	1002	9.0	24.4
5 atm. CO + 1 atm. air	15	1794	10.4	5 atm. N_2 + 1 atm. air	3600	8.4	23.8
5 atm. CO + 1 atm. air	30	3000	12.3	5 atm. N_2 + 1 atm. air	1800	9.4	30.9
5 atm. CO + 1 atm. air	30	600	11.8	5 atm. N_2 + 1 atm. air	1002	9.0	31.0
5 atm. CO + 1 atm. air	45	2712	13.9	5 atm. N_2 + 1 atm. air	3000	9.5	46.3
5 atm. CO + 1 atm. air	60	600	15.3	5 atm. N_2 + 1 atm. air	1002	9.0	70.0
5 atm. CO + 1 atm. air	120	2196	16.1	5 atm. N_2 + 1 atm. air	1200	9.5	70.0
5 atm. CO + 1 atm. air	240	2232	14.8	5 atm. N_2 + 1 atm. air	1800	9.2	60.9
2 atm. CO + 2 atm. O_2 + 1 atm. air	60	2742	10.3	2 atm. CO + 2 atm. N_2 + 1 atm. air	3600	13.9	−25.9
2 atm. N_2 + 2 atm. O_2 + 1 atm. air	60	3672	10.4	4 atm. N_2 + 1 atm. air[b]			16.9
4 atm. O_2 + 1 atm. air	60	3000	11.3	4 atm. N_2 + 1 atm. air	3000	9.0	25.6

[a] This preparation was not irradiated.

[b] This preparation was lost. The per cent increase is calculated on the basis of the value below, since the gas composition of the chambers was the same in both cases.

and pressure of the experimental gas mixture superimposed upon the one atmosphere of air initially present in the chamber. The second column states the duration of exposure prior to irradiation. The third column tabulates the number of chromosomes counted; the fourth indicates the number of dicentrics and centric rings in the experimental preparations. The fifth column records the composition of the gas superimposed on the air in the control chamber. In the sixth and seventh columns the number of chromosomes counted and the per cent dicentrics and centric rings found in the control chamber are listed. In the eighth column is tabulated the per cent increase in dicentrics and centric rings of the experimental preparation when compared with the control preparation. In all experiments except the first one listed, the exposure was 40 r/min. for 10 minutes. In the first experiment tabulated, inflorescences were not irradiated. These results may be summarized as follows:

TABLE 2

FREQUENCIES OF DICENTRICS AND CENTRIC RINGS INDUCED IN TRADESCANTIA MICROSPORES COMPRESSED WITH MIXTURES OF AIR, NITROGEN AND CARBON MONOXIDE EVACUATED, EXPOSED TO AIR AND THEN X-RAYED IN AIR. ALL DOSAGES ADMINISTERED AT A CONSTANT INTENSITY OF 40 R/MIN. FOR 10 MINUTES

(For further discussion, see text)

EXPERIMENTAL				CONTROL			
GAS MIXTURE	DURATION OF EXPOSURE TO GAS, MIN.	CHROMOSOMES COUNTED	PER CENT DICENTRICS AND RINGS	GAS MIXTURE	CHROMOSOMES COUNTED	PER CENT DICENTRICS AND RINGS	PER CENT INCREASE OF DICENTRICS AND RINGS OF EXPERIMENTALS OVER CONTROLS
5 atm. CO + 1 atm. air	60	3000	9.0	5 atm. N_2 + 1 atm. air	3000	9.0	0

1. Irradiation in the presence of positive pressures up to five atmospheres of an indifferent gas such as nitrogen superimposed on one atmosphere of air does not affect the frequency of chromosomal aberrations.

2. Neither high pressures of CO nor high pressures of N_2 cause chromosomal aberrations in the absence of x-radiation.

3. Exposure to CO during irradiation increases the aberration frequency.

4. This increase in aberration frequency depends on the time of exposure to CO prior to irradiation—the longer the exposure time the greater the increase in aberration frequency, with a maximum reached after one hour of prior exposure (figure 1).

5. The CO-induced increase in aberration frequency is almost completely prevented when O_2 is present simultaneously in high concentration.‡

6. Compression with oxygen causes a slight though significant increase in aberration frequency.

Table 2 shows the results of pretreatment with CO on the frequency of x-ray induced aberrations. In this experiment the inflorescences were compressed with five atmospheres of CO for 60 minutes. The chamber was then successively evacuated and filled with air three times to remove the CO. The material was then irradiated as in the previous experiments, the chamber now being filled only with one atmosphere of air. The results of this experiment suggest that:

7. CO must be present *during* irradiation in order for it to have an effect on aberration frequency.

In view of the magnitude of the observed effects, the data presented strongly suggest that CO, when applied simultaneously with irradiation, has a pronounced influence in increasing the radiosensitivity of this material, as measured by chromosomal aberration frequency.

These effects of CO may be analyzed on two different levels. On the

TABLE 3

FREQUENCIES OF DICENTRICS AND CENTRIC RINGS INDUCED IN TRADESCANTIA MICROSPORES EXPOSED TO X-RAYS IN AIR AND THEN COMPRESSED WITHIN 30 SECONDS WITH MIXTURES OF AIR, NITROGEN AND CARBON MONOXIDE. ALL DOSAGES ADMINISTERED AT A CONSTANT INTENSITY OF 40 R/MIN. FOR 10 MINUTES

(For further discussion, see text)

EXPERIMENTAL				CONTROL			
GAS MIXTURE	DURATION OF EXPOSURE TO GAS, MIN.	CHROMOSOMES COUNTED	PER CENT DICENTRICS AND RINGS	GAS MIXTURE	CHROMOSOMES COUNTED	PER CENT DICENTRICS AND RINGS	PER CENT INCREASE OF DICENTRICS AND RINGS OF EXPERIMENTALS OVER CONTROLS
5 atm. CO + 1 atm. air	60	3000	7.8	5 atm. N_2 + 1 atm. air	3000	8.9	Negligible

cytological level we may inquire whether CO causes an increase in the initial frequency of x-ray induced breaks, or affects the reunion of broken ends such that recombination is favored over restitution or both. On the molecular level we may inquire as to the biochemical action of CO on the microspores. Let us first consider the cytological question.

The experimental results thus far presented provide no basis for deciding whether CO acts on the initial frequency of chromosome breakage or on the recovery process. To this end use was made of Lea's calculation that the average time elapsing between breakage and restitution in Tradescantia chromosomes was four minutes.[11] If CO exerted its effect on the recovery process, then it should be possible to affect the yield of chromosome aberrations by exposing inflorescences to CO within this four-minute period. The results of experiments carried out to test this point are presented in table 3. Here the inflorescences were compressed with 5 atmospheres of CO or N_2 within 30 seconds after irradiation. In view of the

absence of effects, it seems evident that CO in some way exerts its action on the breakage process itself. This conclusion finds analogy in the conclusions of Giles and his coworkers on the effects of oxygen during irradiation. They suggest that "the average restitution time for broken ends is the same in the presence and in the absence of oxygen" and that "oxygen exerts its effect by increasing the initial frequency of breaks rather than by modifying the behavior of broken ends during the recovery process."[8]

We may now inquire as to the mode of action of CO within the microspores. Analogous effects have been extensively studied in yeast, in animal tissues and, to a certain extent, in the tissues of plants (Lemberg and Legge,[12] Warburg,[13] and Bhagvat and Hill[14]). There is a substantial evidence that only one group of substances is affected by CO in living systems (exclusive of certain bacteria). These are the heavy-metal-containing enzymes, such as cytochrome oxidase, certain polyphenol oxidases and certain peroxidases. The role of these enzymes in plants and their sensitivity to CO is described in the three references listed above and in papers by Okunuki[15] and Minima and Tylkina.[16]

Okunuki[15] has shown that CO and KCN inhibited the respiration of the pollen of *Lilium* and that the CO inhibition is reversed by light. This last fact gives assurance that cytochrome oxidase is the enzyme affected by CO, since part of the definition of cytochrome oxidase includes light reversibility of CO inhibition. Moreover, this same investigator demonstrated spectroscopically the presence of absorption bands of cytochromes *a*, *b* and *c* in whole pollen and suggested that the cytochrome system played a fundamental role in the normal metabolism of pollen. Since Tradescantia is also a member of the Order Liliales, it seems likely that in Tradescantia microspores the CO acts by inhibiting cytochrome oxidase and that the increased chromosome aberration is a consequence of this inhibition. This suggestion is supported by the observation that oxygen can reverse the CO inhibition (see table 1)—a further distinguishing property of cytochrome oxidase.

The results of the present study may be compared to advantage with other experiments in the literature. Our experiments have shown that CO alone does not cause chromosome breaks. However, other cytochrome poisons, *per se*, such as sodium azide (Wyss, *et al.*[17] on *Staphylococcus*) and KCN (Wagner, *et al.*,[18] on Neurospora) have been shown to cause mutations. It is probable that these agents are exerting their mutagenic effect, not by their action on the cytochrome, but by inhibiting catalase (Sparrow[4]). This is presumed to cause an accumulation of organic peroxides which in some way are thought to be responsible for chromosome breaks and mutations. It is, therefore, noteworthy that CO does not inhibit catalase and consequently does not cause chromosome aberrations in so far as we have been able to determine.

The action of KCN on irradiation has been studied by D'Amato and Gustafsson[19] on barley seeds pretreated with KCN for 24 hours. They found an *increase* in visible mutation rate with low concentrations of KCN (10^{-3} *M*, 10^{-4} *M*), but a *decrease* in visible mutations with 10^{-2} *M* KCN. This apparent inconstancy can be removed if one notes that in the same paper they reported a decrease in mutation rate at high pH (pH 10). Since 10^{-2} KCN has a pH of about 10.5, one may suggest that this high concentration of KCN was exerting its effect in its capacity as a base, rather than as a specific metabolic inhibitor. With this consideration the results of D'Amato and Gustafsson do not conflict with our findings. Moreover, while it is true that KCN is a cytochrome poison by virtue of its heavy-metal-complexing capacity, it is also an effective carbonyl binding agent reacting with many vital substrates (e.g., oxalacetate, pyruvate, etc.). It is thus somewhat difficult to compare results obtained with this relatively non-specific agent (KCN) with results obtained with an agent whose biochemical target is well defined (CO). Therefore, only with reservations do we suggest that KCN acts on x-ray induced mutations in the same way that CO acts on x-ray induced chromosome aberrations—namely, by inhibiting the cytochromes. We are at present studying the effects of HCN gas on x-ray induced chromosome aberrations in Tradescantia.

Since CO acts on the oxygen utilizing part of the metabolic machine, the influence of oxygen *per se* on radiation effects is worth examining. As was noted in the introduction, oxygen has been shown to cause an increase in chromosomal aberrations. We have confirmed this. However, in our experiments the effects of even four atmospheres of oxygen are small compared to effects of CO (see table 1). Notwithstanding, we are confronted with the striking fact that while oxygen alone and CO alone increased aberration frequency, when the two gases were applied simultaneously the CO effect seemed to disappear. The aberration frequency under such circumstances was only slightly increased and was the same as if oxygen alone had been applied (table 1).

To explain this phenomenon we considered the possibility that inhibition of cell respiration by CO caused an *increase* in intra-cellular oxygen tension, and that this in turn caused an increase in chromosome aberrations. However, since even extremely high pressures of oxygen did not exert an appreciable effect in our experiments we have discounted this possibility. Another explanation was suggested by the detection of Okunuki[15] of flavoproteins in *Lilium* pollen. These non-metallo-enzymes can transfer electrons to the cytochromes which then react with molecular oxygen yielding H_2O. However, some flavoproteins can also react directly with molecular oxygen and in so doing yield H_2O_2 as their normal end product (Lardy[20]). It is probable that when the cytochromes are inhibited the

flavoproteins act as terminal oxidases. This could bring about an accumulation of H_2O_2 which would sensitize the chromosomes to irradiation.

It is also worth noting that while cytochromes work at full capacity at low oxygen tensions (*ca.* 4 mm. Hg), flavoprotein activity increases with oxygen tensions at least as high as 760 mm. Hg (one atmosphere) (James and Beevers[21]). With high oxygen tensions we may therefore expect greater flavoprotein activity and consequently more H_2O_2. This would explain the observation of Conger and Fairchild[10] that pure oxygen sensitizes Tradescantia microspores to irradiation and can in fact *per se* produce chromosomal aberrations.

The final consideration is whether the CO is acting directly on the nucleus or indirectly via the cytoplasm. The status of enzymes in the nuclei of cells is not as well defined as that of the cytoplasm and the cytoplasmic granules and this question must be left unanswered for the moment. However, there is one piece of data presented in this paper which suggests that the CO is acting on the cytoplasm. This is the fact that it requires one hour for the CO to exert its full effect in increasing chromosomal aberrations (Fig. 1). It is possible that this delay is due to the time it takes the changes brought about in the cytoplasm by CO to affect the nucleus. Differential centrifugation and direct spectroscopic examination of separated parts of the microspores might yield some direct evidence on the presence of a CO-heavy-metal complex in the nucleus or cytoplasm.

In conclusion we tentatively suggest the following explanation for some of the known effects of oxygen and CO on chromosome aberrations. Our basic premise (admittedly one that is not universally accepted) is that chromosome aberrations closely follow H_2O_2 production.

We believe that CO affects a heavy-metal-containing enzyme in the microspores, probably cytochrome oxidase, in the cytoplasm, nucleus, or both. Furthermore, we suggest that when cytochrome oxidase is inhibited by CO, the flavoproteins act in a greater measure than usual as terminal oxidases, producing large amounts of H_2O_2. This H_2O_2 sensitizes the chromosomes to irradiation. We believe that oxygen, on the other hand, affects the flavoproteins in the microspores: low oxygen tensions decrease their activity as terminal oxidases and this reduces the production of H_2O_2; high oxygen tensions increase their activity, and this increases the production of H_2O_2. Oxygen when applied simultaneously with CO almost completely reverses the effect of CO on chromosome aberrations presumably by reversing the CO-inhibition of cytochrome oxidase.

We would like to emphasize that these suggestions do not preclude the possibility of alternative actions of O_2 during irradiation such as aiding in the production of activated water, etc. However, it seems that in a large measure oxygen exerts its action by altering the biochemical processes of the cell. At present experiments are being carried out to determine the

effects of CO and oxygen on x-ray induced mutations in Drosophila and on x-ray induced injury to cells in tissue culture. It is hoped that these experiments will give some additional clues as to the relation between the metabolic activity of cells and their sensitivity to irradiation.

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‡ The slightly elevated aberration frequency that remains when CO and O_2 are simultaneously present at equal positive pressures has been shown to be due to O_2 alone, and not to incomplete reversal of the CO-cytochrome oxidase complex.

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