SOME EFFECTS OF NITRIC OXIDE AND OXYGEN ON DOMINANT LETHAL PRODUCTION IN X-IRRADIATED DROSOPHILA VIRILIS MALES^{1,2}

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NITRIC oxide (NO) increases X-radiation damage approximately the same amount as equal quantities of oxygen in *Shigella flexneri* (HOWARD-FLANDERS 1957), *Vicia faba* (KIHLMAN 1958), ascites tumor cells (GRAY, GREEN, and HAWES 1958), and barley seeds with greater than 12 percent water content (SPARRMAN, EHRENBURG, and EHRENBURG 1959). These observations, as well as later comparisons of these gases in modifying X-radiation damage (HOWARD-FLANDERS and JOCKEY 1960), have led these authors to conclude that oxygen and nitric oxide are equivalent in their ability to enhance X-radiation damage.

POWERS and his coworkers (POWERS, KALETA, and WEBB 1959; POWERS, WEBB, and EHRET 1959; POWERS, WEBB, and KALETA 1960) have shown that nitric oxide can protect dry *Bacillus megaterium* spores against X-radiation damage, while the irradiation of the spores in similar amounts of oxygen increased the damage observed. Small amounts of nitric oxide can also protect bacteriophage in suspension against X-radiation damage (HOWARD-FLANDERS and LEVIN 1961; COTTON, RINEHART, and PETRUSEK, in preparation).

Nitric oxide appears to have at least two major effects in *Drosophila* males (CAPPS 1961). Three to four percent nitric oxide: (1) delays spermatogenesis for 4 to 6 days in *Drosophila virilis*, and (2) increases dominant lethality in *D. virilis* and also the percentage of sex-linked recessive lethals in X-irradiated *Drosophila melanogaster*. Since 3 percent oxygen did not give the increase in dominant lethality whenever 3 percent nitric oxide did, CAPPS concluded that these gases were operating, at least in part, by separate mechanisms. The interpretation of these nitric oxide experiments in *Drosophila* is difficult, however, because of the delay in spermatogenesis associated with 3 to 4 percent NO. This delay is caused by a lack of sperm leading to nonfertilization and subsequent nondevelopment of some eggs and can not be distinguished from the nondevelopment of eggs fertilized

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with sperm carrying a dominant lethal. Further, the internal oxygen concentration in a respiring cell is often less than the external concentration (KIHLMAN 1958), making it difficult to know if the internal amount of oxygen reported by CAPPS was really equivalent to the amount of nitric oxide that was able to penetrate the cells.

This paper first presents data on dominant lethality from experiments in which *D. virilis* males were irradiated in amounts of nitric oxide that did not appear to cause a significant decrease in the amount of sperm available for fertilization. Second, the amount of dominant lethality due to the presence of small quantities of nitric oxide during the period of irradiation is compared with the dominant lethality curves derived from flies irradiated in a similar amount of oxygen, but under conditions in which metabolic activity was greatly reduced. Third, consideration is given to the reaction between nitric oxide and possible residual amounts of oxygen within the cell leading to the production of nitrous or nitric acid, and perhaps through the action of these products, an increase in the damage observed. Finally, a postirradiation effect of nitric oxide in *D. virilis* males is demonstrated.

MATERIALS AND METHODS

D. virilis males from Texmelucan, Mexico (University of Texas strain #1801.1) were used as the stock irradiated in all experiments. Treated males were mated with heterozygous virgins from a cross between Brazil females (strain #2207.1) and Argentina males (strain #1999.9). The mating procedure is based upon techniques previously discussed by CHANG, WILSON, and STONE (1961). The Texmelucan males were collected from a 3-hour hatching period, and 19 to 24 hours after eclosion were treated with the specific gas mixture, temperature, and X-ray dose for each test. In all experiments the males were pre-treated with a continuous flow of helium for 10 minutes to remove any air from the gas-tight treatment chamber used and to lower the amount of cellular oxygen in the flies. One, two, or three percent nitric oxide or oxygen, carried in helium, was used as the test gas. Since no methods are available for determining the internal gas concentrations of the flies, the test-gas mixture indicated in each experiment refers to the environmental gas concentration of the flies during irradiation. This gas mixture was substituted for the helium and was allowed to equilibrate for 15 minutes prior to the X-irradiation. Inlet and outlet valves on the irradiation chamber were constructed so that this replacement could be brought about without allowing any air to enter the chamber. At the end of the 15-minute test-gas treatment period, the flies were irradiated with the dose of X rays specific for each test. In all experiments the flies were held in the test gas for an additional 15 minutes, except in two experiments (Figure 1g, curves 22 and 23) where one atmosphere oxygen was used as a posttreatment.

After treatment, the flies were matured with three heterozygous females for five days on standard *Drosophila* banana food. *D. virilis* males do not produce mature sperm until the sixth day after eclosion. On the sixth day the males were

remated to three fresh heterozygous virgins. This mating lasted two days and comprised mating period A. The males were remated individually to three fresh heterozygous virgins every two days giving rise to mating periods B, C, D, etc. One or two females from each mating were transferred to fresh food every day for four days, and the number of eggs laid during the previous 24 hours was counted. The number of pupae developing from these vials was scored and a ratio of the number of pupae developing to the total number of eggs laid gave a measure of the amount of dominant lethality under the various test conditions, the parameter of X-radiation damage used.

The X rays used in these tests were administered from a Westinghouse Quadrocondex constant potential machine operating under the following conditions: 250kv, 15ma; $\frac{1}{2}$ mm copper and 1 mm aluminum filtrations; dose rates, (checked before each test with a Victoreen r meter), 390 to 400r per minute. All gases were obtained from the Matheson Company (Nitric oxide, 99.6 percent pure, with 0.37 percent CO_2 as major contaminant; oxygen 99.5 percent pure, with 0.45 percent argon as major contaminant; helium 99.99 percent pure).

RESULTS

The data showing the dominant lethal rates, that is, the percentage of eggs from each mating period that failed to develop into pupae, are presented in Figures 1a through 1g. Included in these figures are the gas mixtures, temperatures, and X-ray doses used in each experiment. The average number of eggs per point that were counted in each experiment is also included with each figure.

Figure 1a, curve 1, shows the dominant lethal rate from *D. virilis* males in one atmosphere air, at room temperature, and with no radiation. This control curve represents the cumulative data of four separate tests. An average value of 7.3 percent nondevelopment was found to be inherent in this system. No correction is made for this in subsequent tests. The increase in dominant lethality caused by 500r of X rays administered while the flies were in one atmosphere of air can also be seen in Figure 1a, curve 2. Included in this figure are the approximate spermatogenic stages sampled at the various mating periods. The results show that spermatids, meiotic stages, and spermatocytes are highly sensitive stages, while spermatogonia and sperm are less susceptible to damage from X rays. This is in agreement with previous studies by ALEXANDER and STONE (1955) and CLAYTON (1962). Since various environmental alterations of the flies have been shown to influence the separation of meiotic stages in this system, no attempt is made to compare the results from individual mating periods. When entire curves from different experiments are compared, however, major differences in both the lethality and in spermatogenic delay can be seen.

Figure 1b demonstrates the effect of 3 percent nitric oxide on the dominant lethality in males that were either unirradiated or irradiated with 500r, 1000r, or 1500r. It is apparent that there is a great deal of nondevelopment of eggs in tests in which 3 percent nitric oxide was used as the radiation environment. No pupae developed from the eggs collected from the early mating periods of these nitric

FIGURE 1.—Effect of various oxygen and nitric oxide tensions, X-ray doses, and temperatures on dominant lethality in *D. virilis* males. Numbers in parentheses are referred to in text—(a) Effect of one atmosphere air. O (1): no radiation, 23° to 25°C, 2,230 eggs/point; Δ (2): 500r, 23° to 25°C, 688 eggs/point.—(b) Effect of 3 percent nitric oxide. O (3): 3 percent nitric oxide, no radiation, 23° to 25°C, 468 eggs/point; Δ (4): 3 percent nitric oxide, 500r, 23° to 25°C.

oxide tests. In an effort to determine if this nondevelopment was due to complete lethality or to a lack of fertilization, males, females, and eggs from the test in which 3 percent nitric oxide and 500r were used as the test conditions were checked for the presence of sperm. No motile sperm were noted in the testes of the treated males until 12 days after eclosion, the time corresponding to the beginning of mating period D. There is an increasing amount of motile sperm produced in older males. An examination of spermathecae of the females mated to these males, and the eggs that they laid during the first three mating periods, showed no sperm. Motile sperm were found in the females and in their eggs only after mating period C, with a greater proportion of the eggs laid by these females containing sperm during later mating periods. Therefore the complete nondevelopment of eggs from mating periods A, B, and C of this test, and a part of the failure of eggs to hatch during later periods, appears to be due to a lack of sperm rather than dominant lethality.

Figures 1c and 1d show the effects of 1 percent and 2 percent nitric oxide on the dominant lethal rates from *Drosophila* males. These curves appear more normal than those obtained with 3 percent nitric oxide. This is reflected in the fact that many eggs that had been laid during early mating periods mature, and the relative X-radiation sensitivity of the spermatogenic stages is in good agreement with control tests. Spot checks of males and females during these early periods showed that apparently normal amounts of sperm were available for fertilization. However, from curves 8 and 12, 1 percent NO and 2 percent NO tests with no radiation, it can be seen that there is a higher than anticipated amount of nondevelopment during mating period A. More expected values are seen in the 1 percent and 2 percent NO tests where the males were irradiated with

467 eggs/point; Inverted triangle (5): 3 percent nitric oxide, 1000r, 23° to 25°C, 260 eggs/point; Half-closed circle (6): 3 percent nitric oxide, 1500r, 23° to 25°C, 116 eggs/point; ■ (7): 3 percent nitric oxide, 500r, 3° to 5°C, 397 eggs/point.—(c) Effect of 1 percent nitric oxide. ○ (8): 1 percent nitric oxide, no radiation, 23° to 25°C, 780 eggs/point; △ (9): 1 percent nitric oxide, 500r, 23° to 25°C, 719 eggs/point; Inverted triangle (10): 1 percent nitric oxide, 1000r, 23° to 25°C, 612 eggs/point; ■ (11): 1 percent nitric oxide, 500r, 3° to 5°C, 661 eggs/point.—(d) Effect of 2 percent nitric oxide. ○ (12): 2 percent nitric oxide, no radiation, 23° to 25°C, 814 eggs/point; △ (13): 2 percent nitric oxide, 500r 23° to 25°C, 738 eggs/point; Inverted triangle (14): 2 percent nitric oxide, 1000r, 23° to 25°C, 682 eggs/point; ■ (15): 2 percent nitric oxide, 500r, 3° to 5°C, 586 eggs/point.—(e) Comparison of dominant lethal rates of *D. virilis* males irradiated in one atmosphere helium and in a gas mixture of 3 percent oxygen and 97 percent helium. ○ (16): one atmosphere helium, 500r, 23° to 25°C, 371 eggs/point; ▲ (17): one atmosphere helium, 500r, 3° to 5°C, 932 eggs/point; Inverted triangle (18): 3 percent oxygen, 500r, 23° to 25°C, 575 eggs/point; ■ (19): 3 percent oxygen, 500r, 3° to 5°C, 659 eggs/point.—(f) Effect of 3 percent nitric oxide plus 3 percent oxygen. ○ (20): 3 percent nitric oxide plus 3 percent oxygen, 500r, 23° to 25°C, 484 eggs/point; ▲ (21): 3 percent nitric oxide plus 3 percent oxygen, 500r, 3° to 5°C, 509 eggs/point.—(g) Reversal of nitric oxide damage by an oxygen post-treatment. ○ (22): 2 percent nitric oxide pretreatment, 1 atmosphere oxygen posttreatment, 500r, 23° to 25°C, 571 eggs/point; Inverted triangle (13): 2 percent nitric oxide, 500r, 23° to 25°C, 738 eggs/point; △ (23): 3 percent nitric oxide pretreatment, one atmosphere oxygen posttreatment, 500r, 23° to 25°C, 248 eggs/point; □ (4): 3 percent nitric oxide, 500r, 23° to 25°C, 467 eggs/point.

500 or 1000r. Perhaps an inactivating reaction of nitric oxide with X-radiation products lowered the amount of this gas available for delaying spermatogenesis, and the high dominant lethal values associated with period A of the nonirradiation tests with 1 percent or 2 percent NO are in part due to a lack of sperm available for fertilization. A further comparison of Figures 1c and 1d shows that the dominant lethal rates are approximately the same when males are irradiated in 1 percent or 2 percent nitric oxide. This is true at either the 500 or 1000r level of X-radiation.

Figure 1e shows a comparison between tests conducted under anoxic conditions (curves 16, 17) and tests performed in a small amount of oxygen (curves 18, 19). It can be seen at lower temperatures, at which the cell cannot remove oxygen from its intracellular environment by metabolism, an oxygen effect is noticed that results in an increase in damage in the presence of 3 percent oxygen. If the experiments were performed using one atmosphere helium as the test gas, or at higher temperatures when 3 percent oxygen was present, when the intracellular oxygen concentration would be low, the radiation damage observed was also low. Simply blocking metabolism of the cells by lowering the temperature has no increased X-radiation enhancing ability (curves 16, 17) where a lower temperature did not alter the X-radiation damage of flies irradiated under anoxic conditions.

The amounts of nondevelopment in the 3 percent nitric oxide tests are not reliable measures of dominant lethality since the nondevelopment of some eggs may be caused by nonfertilization, and since there is a delay in spermatogenesis associated with this amount of NO. Therefore the damage associated with *Drosophila* males treated with 3 percent oxygen and a low temperature that decreases metabolic activity must be compared with the more reliable tests in which 1 percent or 2 percent nitric oxide was used as the test gas (Figure 1c, curve 9; Figure 1d, curve 13). When the entire curves derived from the different test conditions mentioned above are compared, it can be seen that there are equivalent amounts of dominant lethality when flies are irradiated in equivalent intracellular amounts of NO and oxygen. No changes in the radiosensitivity associated with 1 percent or 2 percent nitric oxide were noted when the flies were irradiated at a lower temperature (cf. curve 11, Figure 1c; curve 15, Figure 1d).

Figure 1f shows the effects of radiation on the flies in the presence of 3 percent oxygen and 3 percent nitric oxide. By irradiating in this gas mixture, which will produce nitrogen dioxide (NO_2) in the system, there is an increase in the nondevelopment of the eggs over that observed when the flies are irradiated under anoxic conditions; however, the damage is much less than that observed in flies irradiated with similar doses of X rays and 1, 2, or 3 percent nitric oxide. If the temperature is lowered during irradiation under conditions that produce NO_2 , there is a still greater increase in the damage observed. This is characteristic of the 3 percent oxygen test, in which the amount of nondevelopment was greater at a lower temperature.

Figure 1g shows the effects of an oxygen posttreatment given after the flies had been irradiated in the presence of 2 or 3 percent nitric oxide. The general trend of both tests shows a level of damage lower than that found without the

posttreatment. Further, a comparison of curves 4 and 23 shows that there may be less of a delay in spermatogenesis with an oxygen posttreatment following the irradiation of the males in 3 percent nitric oxide, in that eggs are fertilized and mature during mating period C.

DISCUSSION

HOWARD-FLANDERS (1957), HOWARD-FLANDERS and LEVIN (1961) and HOWARD-FLANDERS and MOORE (1958) working with the dysentery bacillus, *Shigella flexneri*, reported that in wet biological systems the effectiveness of either oxygen or nitric oxide may lie in their ability to react with radicals formed within the irradiated cells. "Vital molecules" necessary for the continued existence of the cell are thought to be equally inactivated by oxy-radicals or NO-radicals. The radiosensitivity of vegetative bacteria (HOWARD-FLANDERS and JOCKEY 1960), bean seeds (KIHLMAN 1958), mouse tumor cells (GRAY, GREEN, and HAWES 1958), and barley seeds at greater than 12 percent water content (SPARRMAN, EHRENBURG, and EHRENBURG 1959) does seem to be affected similarly by equal amounts of either nitric oxide or oxygen. The results presented in the present paper demonstrate that when the metabolic activity of *Drosophila* males is lowered so that little oxygen is enzymatically removed, there is a comparable increase of damage associated with equivalent amounts of nitric oxide or oxygen. This conclusion is contrary to a previous report (CAPPS 1961), in which differences were reported. The differences must have resulted, however, from the fact that under conditions of normal respiration the intracellular concentration of oxygen was lower than in the NO tests.

No methods were available for determining whether residual amounts of oxygen were retained within the irradiation system, or within the males, used during the present nitric oxide tests. Therefore we investigated the effects of nitrous or nitric acid, the ultimate products of the reaction between oxygen and nitric oxide in a water medium, on the radiosensitivity of the cells sampled. Since we excluded as much oxygen as possible, the amount of NO₂ should have been much less in the NO tests than in experiments 20 and 21, which were specifically designed to supply larger amounts of NO₂ to the *Drosophila* during irradiation.

The amount of damage observed when the flies were exposed to this larger amount of NO₂ was only slightly higher than was noted when they were irradiated in helium alone. The developmental delay associated with 3 percent NO is not apparent when 3 percent oxygen is added to the test system. In the NO₂ experiments, when the temperature was lowered to 3° to 5°C, more dominant lethality occurred than when the flies were irradiated at room temperature. This increase in damage at a low temperature is similar to the increase noted in Figure 1e, curve 19, 3 percent oxygen in 97 percent helium, 3° to 5°C and 500r. Since there would have been an excess of oxygen in the NO-NO₂-O₂ gas mixture, the increase in damage noted would be a predicted result if the metabolic removal of the oxygen was not hindered by the NO₂ at the higher temperature. There is no indication at the present time that NO₂, or products arising from NO₂ in this

system, play a major role in increasing X-radiation damage, in causing a developmental delay, or in decreasing metabolic activity in *D. virilis* males. It does appear that a reaction between oxygen and NO reverses the ability of both gases to increase radiation damage, presumably by effectively removing the gases from the system.

Methods were not available at the time of these tests for posttreating the *Drosophila* with NO gas mixtures soon enough after irradiation to be useful. However, oxygen could be substituted immediately after irradiation of the flies in the nitric oxide. As was seen from the results of Figure 1g, treating the flies with oxygen immediately after their irradiation in nitric oxide lowered the amount of damage observed. It does appear from these results that nitric oxide is active after irradiation in enhancing the X-ray damage in *D. virilis* males. Oxygen has no posttreatment effect upon irradiated *Drosophila* (ALEXANDER and STONE 1955; and SCHMID 1961). This postirradiation effect of NO in these flies appears to be another separation of the actions of NO and oxygen in modifying X-irradiation effects in biological organisms. The nonequivalence of these gases in their reactions upon X-irradiated systems has been described previously by POWERS, WEBB, and EHRET (1959) who reported that NO appeared to inactivate long-lived free radicals in dry bacterial spores, while an oxygen reaction with these radiation induced radicals was toxic to the cells. DALE, DAVIES, and RUSSELL (1961) reported another separation of the actions of NO and oxygen. They found that 1 millimole nitric oxide had a latent effect when given as a pretreatment gas that was completely removed from the medium prior to anoxic irradiation of *S. flexneri*. The tentative hypothesis was that some fixation of NO by unknown cell constituents during the pretreatment period led to the increased sensitivity observed. LYNCH and HOWARD-FLANDERS (1962) later confirmed the pretreatment effect of 1 millimole nitric oxide previously reported by DALE *et al.* (1961). Furthermore, since the level of sulfhydryl compounds was found to be less after a pretreatment with nitric oxide, and since cysteine given prior to irradiation reversed much of the nitric oxide pretreatment effect, they felt that one action of this gas might be to lower the amount of cellular sulfhydryl compounds available to donate hydrogen atoms to free radical DNA molecules. Another action of nitric oxide, they felt, might be the reaction of the gas with DNA radicals, which could lead to DNA incapable of further biological activity. Perhaps one action of an oxygen posttreatment in this *Drosophila* system is to break such inactivating NO complexes. Our data do not distinguish whether a reaction occurs of oxygen with free (or bound) NO, or if some other mechanism is at work in reversing the latent nitric oxide effect.

SUMMARY

The ability of small amounts of nitric oxide (NO) or oxygen to increase dominant lethality was tested in X-irradiated *Drosophila virilis* males. The data support the following conclusions: (1) When physiological conditions are adjusted so that equal amounts of either NO or oxygen are present within the spermatog-

genic cells being tested, equal amounts of either gas cause equivalent increases in the damage. (2) When flies were irradiated in the presence of NO and oxygen, which will react to form NO₂, much of the effect of both gases to increase X-ray damage was reversed. Therefore, neither NO₂ nor its hydrolysis products nitric or nitrous acid appear to be the major mutagens in this system. (3) An oxygen posttreatment given to *D. virilis* males immediately after their irradiation in 2 or 3 percent NO reverses some of the damage. Nitric oxide appears to be modifying the survival of these males after irradiation.

The relations of these data to other investigations concerning the possible actions of NO in altering X-ray damage is discussed.

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