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*MUTAGENIC EFFECTS OF HIGH OXYGEN TENSIONS ON ESCHERICHIA COLI**

By W. O. Fenn, R. Gerschman, D. L. Gilbert,
D. E. Terwilliger, and F. V. Cothran

DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY, ROCHESTER, NEW YORK

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It has been postulated that there exists a common mechanism operating in the biological effects of oxygen and X-irradiation.[1] In further support of this theory it seemed worth while to determine whether high pressure of oxygen would cause mutations in *Escherichia coli* similar to those caused by radiation and demonstrated by Demerec.[2] Conger and Fairchild[3] demonstrated chromosome aberrations in *Tradescantia* caused by high oxygen pressures which were similar to those caused by X-rays. For this purpose Dr. Demerec kindly supplied us with a streptomycin-dependent strain (B/Sd-4) and furnished detailed descriptions of the method which he has employed.[4]

1. Heart infusion broth, containing 10 μg. streptomycin/ml, was inoculated with *E. coli* (B/Sd-4) and placed on a shaker for approximately 22 hours at 37° C.

2. After incubation, 100–150 ml. of the broth-bacterial culture were transferred to a centrifuge bottle. After centrifugation the supernatant was discarded, and the bacteria were washed in sterile saline to remove streptomycin and carefully resuspended in saline.

3. From this suspension, 0.15-ml. samples were spread on streptomycin-free agar plates for a count of the number of spontaneous mutants. From the same suspension a series of dilutions in sterile saline was made, each member of the series having one-fifth the concentration of the preceding one. Four 0.15-ml. aliquots of each of these dilutions (in the countable range) were spread on agar plates containing 10 μg. of streptomycin/ml. Colonies counted on these plates gave the number of viable bacteria of the streptomycin-dependent or "normal" strain prior to the experimental treatment.

4. Two 40-ml. samples of the suspension prepared under 2 were transferred to sterile centrifuge bottles or flasks which were placed in identical steel compression chambers at room temperature. One of these chambers was left at atmospheric pressure in air, while to the other was added pure oxygen to a total pressure of 4, 6, 8, or 10 atm. in addition to the oxygen present in the original air in the chamber. The latter chamber was left attached to the oxygen tank through a reducing gauge, so that the pressure was maintained at the desired level in spite

of small leaks during the night (about 16 hours). Generally, the temperature in the tanks was about 2° C. lower than the room temperature. After the exposure period the pressure was reduced slowly to avoid formation of bubbles.

5. Following the treatment, counts were made for both mutants and non-mutants in both samples according to the technique outlined in 3. Plates were incubated at 37° C. and counts were made after 24, 48, and 72 hours. All calculations were based on the final readings. Growth of the original B/Sd-4 strain was essentially complete after 24 hours, but only 20 per cent of the mutants had appeared in the same length of time. After 48 hours the growth of the mutants was about 90 per cent complete. Ryan[5] has described a similar delay in the appearance of mutants.

Results.—A preliminary series of experiments was carried out in the summer of 1955 (by D. T.). The first 3 experiments are omitted because of some differences in technique, and the remaining 19 experiments are summarized in Table 1. In

TABLE 1

COUNTS OF NORMAL (N) AND MUTANT (M) FORMS OF *E. coli* AFTER TREATMENT WITH 6–10 ATM. O_2 FOR 16 HOURS AS COMPARED TO TREATMENT WITH 1 ATM. OF AIR FOR SAME PERIOD

(Averages of 19 Experiments, with Standard Errors)

	$N \times 10^8$	M	$M/N \times 10^{-8}$	$\frac{M/N \text{ in } O_2}{M/N \text{ in Air}}$
Air	16.6	43.4	3.4 ± 0.055	1.55 ± 0.16
O_2	14.8	47.1	4.7 ± 0.86	

this series no counts were made on the suspension prior to treatment, so the table includes only the counts made on the suspensions after exposure to air or 6–10 atm. of O_2 for about 16 hours. Actual counts are given for the number of normal streptomycin dependent (N) and for the mutant or streptomycin-independent (M) strains, as well as the number of mutants per 10^8 normal bacteria (M/N). It is seen that the number of mutants per 10^8 of viable bacteria increased from 3.4 to 4.7 as a result of the oxygen treatment. While the difference between these two average figures is not significant, on account of the wide variations between the densities of the suspensions in the different experiments, a significant result is obtained by calculating for each experiment the ratio of the M/N value for O_2 to the M/N value for air. The average result in the last column shows that the treatment with oxygen increased the number of mutants per 10^8 viable bacteria 1.55 ± 0.16 times. This value differs from 1 with a $P = 0.3$ per cent.

Treatment with oxygen tended to kill or prevent the growth of the normal strain by 10 per cent ($N_{O_2}/N_{air} = 0.90$), while it increased the growth of the mutant strain by 25 per cent ($M_{O_2}/M_{air} = 1.25$). These differences are significant at the 5 per cent and 2.2 per cent levels, respectively. Decreased growth of the normal strain is only partly responsible for the increased number of mutants in O_2 per 10^8 of viable bacteria. The data suggest also an absolute increase in the number of mutants as a result of the oxygen treatment.

A second series of experiments was carried out in the spring of 1956 by B. Ruhm and D. Caccamise. The purpose of these experiments was to demonstrate more clearly that the oxygen produced an absolute increase in the number of mutants. To this end, counts were made in the suspension *before* it was exposed to oxygen

or air in addition to the counts made afterward. The results in Table 2 show that the number of normal streptomycin-dependent bacteria (N) was little affected by treatment with either air or oxygen but the number of mutants (M) was increased from 66.7 to 129.5 per ml. by treatment with oxygen, while similar treatment with air had no appreciable effect. This is the essential finding, but the results can be analyzed in other ways to the same end. Thus the figures show that the number of mutants per 10^8 of normal bacteria (M/N) was increased on the average 2.44 ± 0.47 times ($P = 0.016$) by O_2 treatment as compared to air. This figure compares with the value of 1.55 ± 0.16 obtained from Table 1. The count of the normal bacteria was increased 16 per cent after treatment in air (perhaps due to additional growth) and was decreased 7 per cent by treatment with 10 atm. of oxygen. The number of mutants was decreased 5 per cent by air treatment (relative to the pretreatment control) but was increased 93 per cent by treatment with oxygen.

TABLE 2

COUNTS OF MUTANTS (M) AND NONMUTANT (N) FORMS OF *E. coli* BEFORE TREATMENT AND AFTER EXPOSURE TO AIR OR 10 ATM. OF O_2 FOR 16 HOURS

	Before			After Air			After O_2			
Exp. No.	N ($\times 10^8$ per Ml.)	M (Per Ml.)	M/N ($\times 10^{-8}$)	N ($\times 10^8$ per Ml.)	M (Per Ml.)	M/N ($\times 10^{-8}$)	N ($\times 10^8$ per Ml.)	M (Per Ml.)	M/N ($\times 10^{-8}$)	$\frac{(M/N)\ O_2}{(M/N)\ \text{Air}}$
1	34.0	41.7	1.23	24.1	36.7	1.52	23.5	81.7	3.48	2.29
2	24.1	78.3	3.24	29.6	78.3	2.64	16.6	162.2	9.77	3.70
3	32.8	75.3	2.30	45.8	96.7	2.11	37.6	431.3	11.50	5.45
4	37.6	61.7	1.64	51.9	40.0	0.77	42.8	105.0	2.45	3.18
5	23.7	58.3	2.46	30.6	78.0	2.55	24.7	118.7	4.80	1.88
6	14.7	53.3	3.63	14.8	53.2	3.80	9.5	65.0	6.84	1.80
7	26.7	56.7	2.12	9.0	46.7	5.19	20.8	61.3	2.95	0.57
8	13.1	73.7	5.60	20.7	75.3	3.63	16.0	53.3	3.33	0.92
9	15.4	101.3	6.58	22.6	56.7	2.51	15.7	86.7	5.52	2.20
Av.	24.7	66.7	3.20	27.7	62.4	2.75	23.0	129.5	5.63	2.44
St. error	±2.8	±5.5	±1.25	±4.2	±6.6	±0.4	±3.4	±0.37	±1.0	±0.47

The arbitrary selection of 6–10 atm. for the experimental O_2 pressure proved fortunate, since higher pressures are progressively more toxic. In 4 additional experiments by J. V. C., counts were made before and after treatment for 16 hours. The normal count was reduced to 54 per cent after exposure to oxygen, while the mutant count rose to 197 per cent. The M/N ratios were, of course, all increased after the treatment with oxygen, but this was partly due to some killing of the normals by oxygen. Nevertheless, the absolute number of mutants was increased by the O_2 treatment.

It must be admitted that there are considerable variations among the different experiments so far presented. Oxygen appeared to increase the number of mutants in 14 out of 19 experiments in Table 1 and in 7 of the 9 experiments in Table 2. Considerable errors are to be expected in a technique of this sort, however, and statistically the data show clearly that high pressure of oxygen does have a mutagenic effect.

The possibility still remains that the data presented may depend upon pressure per se rather than upon the partial pressure of the oxygen itself. To answer this question, we have tried a number of experiments with nitrogen in place of oxygen, and these results are presented in Table 3. The first 3 experiments were done

TABLE 3

NITROGEN CONTROLS: COUNTS OF NORMAL (N) AND MUTANT (M) FORMS OF *E. coli* BEFORE AND AFTER TREATMENT WITH AIR OR AIR PLUS 10 ATM. N_2
(Average Values and Standard Errors)

	N ($\times 10^8$)	M	M/N ($\times 10^{-8}$)
3 experiments:			
After air	14.6 ± 2.0	33.4 ± 6.2	2.25 ± 0.14
After N_2 + air	16.4 ± 2.5	22.7 ± 2.2	1.48 ± 0.13
6 experiments:			
Before treatment	29.1 ± 2.0	53.6 ± 5.0	1.89 ± 0.24
After air	28.0 ± 3.0	59.1 ± 7.8	2.15 ± 0.25
After N_2 + air	25.4 ± 2.8	62.0 ± 7.5	2.54 ± 0.31

along with the experiments of Table 1 and lacked a count prior to the overnight treatment with air or nitrogen (10 atm.) plus air. In this case the nitrogen caused, if anything, a decrease in the number of mutants. This may, however, have been due to anoxia, since the chambers leaked to some extent and allowed the nitrogen to flush out some of the oxygen originally present in the chamber. Another 6 experiments were carried out later in which 2–3 per cent of oxygen was added to the nitrogen to avoid this complication. The averages of these experiments show no significant difference between the bacteria exposed to air alone and those exposed to air plus 10 atm. of nitrogen. Likewise, the counts are the same before and after treatment within the limits of the experimental error. It is concluded that pressure per se does not influence mutagenesis.

It has been shown by Jordan, Mefferd, and Wyss[6] that *E. coli* can be protected against the toxic effects of radiation by treatment with pyruvate. Gerschman, Gilbert, *et al.*[1] have shown that certain substances which protect against radiation are also protective against oxygen poisoning. For this reason we tried adding pyruvate to our cultures of *E. coli* to find out whether this prevented the mutagenic effect of high oxygen pressures. No counts prior to treatment were made, but 3 suspensions were set up: (1) without pyruvate but exposed to oxygen, (2) with pyruvate and exposed to oxygen, and (3) with pyruvate but exposed only to air. The average results of 6 experiments (by F. V. C) are shown in Table 4. Com-

TABLE 4

ELIMINATION OF THE MUTAGENIC EFFECT OF OXYGEN BY PYRUVATE*

	N (Per Ml. $\times 10^8$)	M (Per Ml.)	M/N (Per Ml. $\times 10^{-8}$)
Oxygen	26.3 ± 4.3	156 ± 37	6.1 ± 1.5
Oxygen + pyruvate	16.4 ± 3.7	38.3 ± 5.6	2.9 ± 0.7
Air + pyruvate	28.0 ± 3.2	55.6 ± 8.7	2.2 ± 0.4

* Counts of normal (N) and mutant (M) forms of *E. coli* after treatment with O_2, with and without pyruvate and after air + pyruvate. Average of 6 experiments with standard errors.

paring suspensions 1 and 2, it is evident that the addition of pyruvate to cultures exposed to oxygen diminished the M/N ratio from 6.1 to 2.9. Comparing suspensions 2 and 3, it appears that the exposure to oxygen instead of air in cultures containing pyruvate caused no significant increase in the M/N ratio. It is concluded from these figures that pyruvate prevents the mutagenic effect of oxygen, just as it decreases the mutagenic effect of radiation in the same organism. Many more experiments of this type are required, but these preliminary results encourage us to believe that further experimentation will support the thesis that there is a certain similarity of fundamental mechanism between radiation and oxygen toxicity.

Discussion.—The experiments reported appear to show that high pressures of oxygen are capable of exerting a mutagenic effect in *E. coli* which is comparable to the mutagenic effect of radiation or of chemical mutagenic agents such as those described by Demerec[2] and Demerec, Bertani, and Flint.[4] Conversely, these same mutations are inhibited by anoxia.[7, 8] As a possible explanation for this similarity between oxygen and radiation, one thinks of chromosome damage by free radicals formed from the effects of radiation on water or formed by high pressures of oxygen in a biological medium where univalent oxidations are taking place.[9] According to Giles,[12] oxygen increases the mutagenic effect of radiation only when it is present during the time of the radiation and because it intensifies the amount of chromosome breakage. Baker and Von Halle,[11] however, believe that oxygen affects the result because it interferes with the subsequent union of the broken ends of the chromosomes. In neither of these papers is there any discussion of the effects of oxygen by itself without any simultaneous radiation. According to Plaine,[10] exposure to oxygen alone produces a condition known as "erupt eyes" and increases the incidence of tumors in *Drosophila melanogaster*. The effect of radiation in producing these same abnormalities is then regarded as "an enhancement of the effect of oxygen tension alone" and is ascribed to the formation of hydrogen peroxide. Formation of free radicals might have a similar effect. On the other hand, Lück[13] has found that treatment of bacteria with compressed oxygen causes increased catalase activity and inhibition of alkaline phosphatase, dehydrogenase, and rate of formation of lactic acid and CO_2. All these enzymic changes might, however, be regarded as secondary to some primary effect such as the formation of free radicals. Holman[14] suggests that the formation of tumors is analogous to the formation of abnormal bacteria and attributes the result to an increase in the concentration of hydrogen peroxide.

In reviewing this work Dr. E. A. Adelburg has pointed out to us the possibility of selective survival in oxygen as another reasonable explanation of our observations. As a result of this suggestion, we tried further experiments with additional counts made prior to the exposure to oxygen. The results show that selective survival is not the only explanation of the figures because we have now demonstrated an *absolute increase in the number of mutants* after exposure to the compressed oxygen as compared to the same culture before treatment. Nevertheless, there is perhaps still room for some reservation. It is conceivable, for example, that the cells might come in clumps and that each clump might act either as a normal or as a mutant, but not both. Then if oxygen tended to kill off the normal cells, more of the clumps might function as mutants. Similarly, each individual cell might have the equivalent of more than one nucleus, some of them normal and some mutant. Oxygen might then promote the selective survival of the mutant "nuclei" and so result in more cells under mutant than under normal control. It might also be supposed that oxygen would tend to unclump more mutants than normal cells, but such highly selective action seems highly improbable. Except for such rather remote possibilities, our experiments appear to demonstrate a true mutagenic effect of oxygen, due presumably to chromosome damage resulting from an increased concentration of free radicals.

Summary.—Using a streptomycin-dependent strain of *E. coli*, it is shown that the number of spontaneous mutants to the streptomycin-independent variety is

increased 1.5–2.4 times by treatment for 16 hours with 6–10 atm. of oxygen. This demonstrates a mutagenic effect of high pressures of oxygen which is comparable to that caused by radiation.

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*MOLECULAR STRIAE FROM AN INDANTHRENE DYE**

BY LOUIS W. LABAW AND RALPH W. G. WYCKOFF

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE
DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE, BETHESDA 14, MARYLAND

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It has recently been demonstrated by Menter[1] that electron micrographs of suitably oriented crystals of copper and platinum phthalocyanins may appear covered by striations separated by the known distance between molecular centers. These observations have also provided a striking demonstration of dislocations and other crystalline faults at the molecular level and are beginning to yield valuable information concerning the interaction between a coherent beam of electrons and a crystalline sample under the conditions requisite to high-resolution electron microscopy. In view of the wide potential usefulness of so direct a method of visualizing the separations of molecules having weights of a few hundreds, we have been examining a number of crystalline organic substances of suitable molecular size, to define more clearly the conditions under which these phenomena appear and to see how they differ from compound to compound. Such observations are needed to