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# Response of *Escherichia coli* to Ferrous Ions. II. Influence of Nitrogen and Oxygen on the Mutagenic and Lethal effects of Fe++ for a Streptomycin-dependent Strain

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# RESPONSE OF ESCHERICHIA COLI TO FERROUS IONS

## II. Influence of Nitrogen and Oxygen on the Mutagenic and Lethal Effects of $Fe^{++}$ for a Streptomycin-dependent Strain

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The response of *Escherichia coli* to treatment with ferrous solution has been found to be more pronounced when the cells are exposed at 1 C instead of at 37 C. The effects are even greater when, in addition, the exposure temperature is changed from 1 C to 37 C during $Fe^{++}$ treatment. With the streptomycin-dependent strain Sd-4, the frequency of reversion to nondependence is increased enormously; and with strain B, the processes of synthesis are inhibited significantly (Catlin, 1953).

In X-ray experiments, a temperature effect was demonstrated by King (1947) and by Sax (1947). They found that the frequency of induced mutation was higher when test material (*Tradescantia* microspores, *Drosophila*) was maintained during irradiation at a temperature near freezing instead of at room temperature. Subsequently, the increased radiosensitivity associated with low temperature irradiation was shown to result from an increased oxygen tension. Baker and Sgourakis (1950) pointed out that the solubility of oxygen in water is higher at colder temperatures and demonstrated that irradiation effects comparable to those produced at low temperature could be obtained directly by irradiating under a high oxygen tension. This finding has been extended to X-ray studies with other organisms, including bacteria (Hollaender, Baker, and Anderson, 1951).

In view of the possible relevance of these studies to the temperature effect associated with $Fe^{++}$ treatment, the influence of nitrogen and of oxygen on the mutagenic and lethal consequences of ferrous treatment was investigated.

### EXPERIMENTAL METHODS

The streptomycin-dependent strain Sd-4 of *Escherichia coli* and the methods employed were similar to those previously described (Catlin, 1953), except for a few additional features. Tests of the mutagenic action of $Fe^{++}$ involved determining the frequency of reversion from streptomycin dependence to nondependence. Aliquots of a washed aqueous suspension of cells were pipetted into sterile tubes, which were placed in either a 1 C or a 37 C water bath. Each tube was closed with a two-hole rubber stopper fitted with glass tubing, one piece of which extended to the bottom of the tube. The cells were bubbled vigorously with streams of water washed nitrogen or oxygen for a period of about 5 minutes before the temperature adjusted solution of $FeSO_4$ was added. Changes of gas or temperature were made without opening the tubes. At the end of the treatment, however, some air reached the cells as they were being diluted or centrifuged. Assays of the streptomycin-dependent survivors and the nondependent revertants were carried out by the previously described procedure.

### RESULTS

Table 1 shows the results of one representative experiment in which aliquots of the same cellular suspension were treated with $Fe^{++}$ in the presence of either oxygen or nitrogen. After an initial 30 minute period of exposure at 1 C, certain reaction tubes were changed to 37 C, and at the same time the stream of nitrogen was replaced with a stream of oxygen (or vice versa). Three major points were established by these data. (1) Whether the ferrous-reaction mixtures were bubbled with nitrogen or with oxygen, exposure at 1 C produced a far greater lethal effect and a higher reversion frequency than exposure at 37 C. Thus, the notion that $Fe^{++}$ exposure might be more effective at 1 C than at 37 C primarily because of increased oxygen tension at the lower temperature was not supported. (2) Treatment with $Fe^{++}$ at 1 C in the presence of oxygen produced a somewhat greater lethal effect than the corresponding treatment in nitrogen. The reversion frequency (number of revertants per $10^8$ survivors) produced by $Fe^{++}$ at 1 C was about the same whether nitrogen or oxygen had been used during the treatment. (3) Strikingly different results were obtained when the 1-to-37 C sequence of $Fe^{++}$ exposure was carried out in nitrogen instead of in oxygen. The use of nitrogen so affected the reversion frequency that the customary increase or "burst" of revertants was not exhibited. Moreover, the fraction of survivors under these conditions was increased greatly. A comparison of survival data for tubes 1 and 3 shows that there were fewer survivors when exposure to $Fe^{++}$ at 1 C was terminated after 30 minutes than when the treatment was extended to include a secondary period at 37 C. In the presence of nitrogen at 37 C, both the lethal and mutagenic effects of $Fe^{++}$ treatment at 1 C could be reversed partially. Some reversal effect could be obtained in nitrogen even when the initial exposure had been carried out in oxygen (tube 9). No reversal was educed, however, when the secondary exposure at 37 C took place in oxygen, even though the initial exposure to $Fe^{++}$ at 1 C had been carried out in nitrogen. Under these conditions, the fraction of survivors and the "burst" of revertants were within the ranges that would have been expected if the treatment had been conducted entirely in air.

These results clearly indicate the importance of differences in oxygen tension during exposure of cells to $Fe^{++}$. However, they do not clarify the question of the difference between nitrogen and oxygen during the brief period of temperature change. On the assumption that the temperature of the reaction mixture would rise from 1 C to 37 C within 2 to 3 minutes after the time the tube was transferred to the higher temperature, tests were conducted in which for one pair of tubes the change in gas was made 28 minutes after the cells and $Fe^{++}$ were mixed, for a second pair the change was made after 33 minutes, for a third pair one gas was substituted for the other only during the 5 minute temperature transition period, for a fourth pair one gas was used throughout; the temperature change being initiated after 30 minutes for all these tubes.

The data from one such experiment are shown in table 2. A conspicuous difference in effect was produced by replacing the nitrogen atmosphere with oxygen for a period of 5 minutes only,

TABLE 1

*Effects of nitrogen and oxygen on response of Escherichia coli, strain B/Sd-4, to $Fe^{++}$ at 37 C and 1 C*

| TUBE | SOLN* | INITIAL EXPOSURE | | | SECONDARY EXPOSURE | | | Sd SURVIVORS | | REVERTANTS PER ML | REVERTANTS PER $10^8$ SURVIVORS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Gas | Temp | Time | Gas | Temp | Time | % | No. per ml | | |
| | | | C | min | | C | min | | | | |
| 1 | $Fe^{++}$ | $N_2$ | 1 | 30 | — | — | — | 34 | $6.7 \times 10^8$ | 1,129 | 169 |
| 2 | $Fe^{++}$ | $N_2$ | 1 | 130 | — | — | — | 15 | $2.9 \times 10^8$ | 686 | 237 |
| 3 | $Fe^{++}$ | $N_2$ | 1 | 30 | $N_2$ | 37 | 80 | 60 | $1.2 \times 10^9$ | 2,600 | 217 |
| 4 | $Fe^{++}$ | $N_2$ | 1 | 30 | $O_2$ | 37 | 60 | 10 | $1.9 \times 10^8$ | 8,567 | 4,509 |
| 5 | $Fe^{++}$ | $N_2$ | 37 | 70 | — | — | — | 50 | $1.0 \times 10^9$ | 850 | 85 |
| 6 | $Fe^{++}$ | $O_2$ | 1 | 30 | — | — | — | 20 | $4.0 \times 10^8$ | 786 | 197 |
| 7 | $Fe^{++}$ | $O_2$ | 1 | 130 | — | — | — | 12 | $2.4 \times 10^8$ | 500 | 208 |
| 8 | $Fe^{++}$ | $O_2$ | 1 | 30 | $O_2$ | 37 | 80 | 5 | $1.0 \times 10^8$ | 1,043 | 1,043 |
| 9 | $Fe^{++}$ | $O_2$ | 1 | 30 | $N_2$ | 37 | 60 | 33 | $6.6 \times 10^8$ | 30,367 | 4,601 |
| 10 | $Fe^{++}$ | $O_2$ | 37 | 70 | — | — | — | 80 | $1.6 \times 10^9$ | 914 | 57 |
| 11 | Water | $N_2$ | 1 | 30 | $N_2$ | 37 | 100 | 95 | $1.9 \times 10^9$ | 800 | 42 |
| 12 | Water | $O_2$ | 1 | 30 | $O_2$ | 37 | 100 | 90 | $1.8 \times 10^9$ | 771 | 43 |
| Initial cellular suspension | | | | — | — | — | — | 100 | $2.0 \times 10^9$ | 780 | 39 |

* $Fe^{++} = 3.0 \times 10^{-5}$ M $FeSO_4$.

during which the 1-to-37 C change was taking place. The presence of oxygen during this short critical period resulted in a "burst" of revertants of the ordinary magnitude and a great decrease in number of survivors, compared with results for the corresponding reaction mixture continuously bubbled with nitrogen. On the other hand, replacing the oxygen atmosphere with nitrogen during a similar 5 minute interval gave results similar to those for the corresponding treatment conducted entirely in oxygen.

TABLE 2

*Effects of changes in atmosphere and temperature during exposure of Escherichia coli, strain B/Sd-4, to $Fe^{++}$*

| TUBE | SOLN* | GAS USED AT VARIOUS PERIODS | | | DURATION OF EXPOSURE TEMP | | TOTAL EX-POSURE | Sd SURVIVORS | | REVERTANTS PER ML | REVERTANTS PER $10^9$ SURVIVORS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0–28 min | 28–33 min | 33 min–end | At 1 C | At 37 C | | % | No. per ml | | |
| | | | | | *min* | *min* | *min* | | | | |
| 1 | $Fe^{++}$ | $N_2$ | $N_2$ | $N_2$ | 35 | — | 35 | 26 | $5.0 \times 10^8$ | 783 | 157 |
| 2 | $Fe^{++}$ | $O_2$ | $O_2$ | $O_2$ | 35 | — | 35 | 15 | $2.9 \times 10^8$ | 567 | 196 |
| 3 | $Fe^{++}$ | $N_2$ | $N_2$ | $N_2$ | 30 | 35 | 65 | 42 | $8.0 \times 10^8$ | 667 | 83 |
| 4 | $Fe^{++}$ | $N_2$ | $O_2$ | $N_2$ | 30 | 35 | 65 | 15 | $2.8 \times 10^8$ | 3,117 | 1,113 |
| 5 | $Fe^{++}$ | $O_2$ | $O_2$ | $O_2$ | 30 | 35 | 65 | 9 | $1.8 \times 10^8$ | 1,150 | 639 |
| 6 | $Fe^{++}$ | $O_2$ | $N_2$ | $O_2$ | 30 | 35 | 65 | 10 | $1.9 \times 10^8$ | 1,171 | 616 |
| 7 | $Fe^{++}$ | $N_2$ | $O_2$ | $O_2$ | 30 | 70 | 100 | 16 | $3.0 \times 10^8$ | 4,814 | 1,605 |
| 8 | $Fe^{++}$ | $N_2$ | $N_2$ | $O_2$ | 30 | 70 | 100 | 23 | $4.4 \times 10^8$ | 3,980 | 905 |
| 9 | $Fe^{++}$ | $O_2$ | $N_2$ | $N_2$ | 30 | 70 | 100 | 31 | $5.9 \times 10^8$ | 11,786 | 1,998 |
| 10 | $Fe^{++}$ | $O_2$ | $O_2$ | $N_2$ | 30 | 70 | 100 | 11 | $2.0 \times 10^8$ | 4,086 | 2,043 |
| 11 | Water | $N_2$ | $N_2$ | $N_2$ | 30 | 90 | 120 | 88 | $1.7 \times 10^9$ | 414 | 24 |
| 12 | Water | $O_2$ | $O_2$ | $O_2$ | 30 | 90 | 120 | 88 | $1.7 \times 10^9$ | 529 | 31 |
| Initial cellular suspension | | | | | — | — | — | 100 | $1.9 \times 10^9$ | 363 | 19 |

* $Fe^{++} = 3.0 \times 10^{-6}$ M $FeSO_4$.

treatment), except where the initial nitrogen atmosphere was maintained until after the temperature change had taken place and then was replaced with oxygen (tube 8). In this case, the reversion frequency was always considerably lower.

The degree to which a bacterial population will recover from the lethal effects of initial $Fe^{++}$ treatment is influenced greatly by the length of time it is retained at 37 C during subsequent $Fe^{++}$ exposure in nitrogen. In the experiment shown in table 3, the only difference in treatment between tubes 2 and 3 was that the latter received 120 minutes of additional exposure at 37 C, both being in nitrogen continuously. The increase in number of survivors from $6.3 \times 10^8$ ($N_1$) to $1.0 \times 10^9$ ($N_2$) represents a relative recovery of 0.37 $\left(\frac{N_2 - N_1}{N_2}\right)$. Similarly, the treatment for tubes 4 and 5 was the same, except that the latter received an additional exposure of 75 minutes. Although the survival level was lower here than in tubes 2 and 3, owing to the 5 minute period of oxygenation, the increase in number of survivors from $9.5 \times 10^7$ to $1.6 \times 10^8$ represents

If the effect of the gas were not related intimately with the temperature change, it would make little difference whether the change from oxygen to nitrogen took place 2 minutes before or 3 minutes after the time that the tube was changed from the cold to the warm temperature. The results for tubes 7 to 10 (table 2) show that there were significant differences in survival. Irrespective of which gas was present during the initial exposure, the number of survivors was higher when nitrogen, rather than oxygen, was present during the period of temperature change. There were only slight differences in reversion frequencies (which were increased by the longer a relative recovery of 0.41. Decreases in reversion frequency were associated with this partial recovery. No recovery resulted in the corresponding oxygen-bubbled tubes.

Recovery of viability on the part of a considerable proportion of cells of strain Sd-4 inactivated by initial 1 C $Fe^{++}$ treatment is presumably a reflection of a general metabolic response. One would expect, therefore, that the treatment procedure which effects partial recovery would increase likewise cellular processes of synthesis. That such is the case was demonstrated by tests of the relative uptake of $S^{35}$, $C^{14}$, and $P^{32}$ by suspensions of the B strain after exposure to $Fe^{++}$. (Methods were similar to those used in part I of this series (Catlin, 1953); data to be presented in a subsequent part.) Furthermore, cells exposed in the presence of nitrogen to $Fe^{++}$ at 37 C showed little or no subsequent inhibition of synthesis; uptake of $S^{35}$, $C^{14}$, and $P^{32}$ being very similar to, or in some cases higher than, the corresponding uptake values for untreated control suspensions.

TABLE 3

*Effects of atmosphere and duration of $Fe^{++}$ exposure at 37 C on the response of Escherichia coli, strain B/Sd-4*

| $Fe^{++}$* TREATMENT TUBE | GAS USED AT VARIOUS PERIODS | | | DURATION OF EXPOSURE TEMP | | TOTAL EXPOSURE | Sd SURVIVORS | | REVERTANTS PER ML | REVERTANTS PER $10^8$ SURVIVORS |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0–33 min | 33–38 min | 38 min–end | At 1 C | At 37 C | | % | No. per ml | | |
| | | | | *min* | *min* | *min* | | | | |
| 1 | $N_2$ | $N_2$ | $N_2$ | 40 | — | 40 | 43 | $5.6 \times 10^8$ | 2,200 | 393 |
| 2 | $N_2$ | $N_2$ | $N_2$ | 35 | 20 | 55 | 48 | $6.3 \times 10^8$ | 6,500 | 1,032 |
| 3 | $N_2$ | $N_2$ | $N_2$ | 35 | 140 | 175 | 77 | $1.0 \times 10^9$ | 4,300 | 430 |
| 4 | $N_2$ | $O_2$ | $N_2$ | 35 | 45 | 80 | 7 | $9.5 \times 10^7$ | 2,129 | 2,241 |
| 5 | $N_2$ | $O_2$ | $N_2$ | 35 | 120 | 155 | 12 | $1.6 \times 10^8$ | 1,986 | 1,241 |
| 6 | $O_2$ | $O_2$ | $O_2$ | 35 | 20 | 55 | 2 | $2.3 \times 10^7$ | 486 | 2,113 |
| 7 | $O_2$ | $O_2$ | $O_2$ | 35 | 140 | 175 | 2 | $2.1 \times 10^7$ | 357 | 1,700 |
| 8 | $O_2$ | $N_2$ | $O_2$ | 35 | 45 | 80 | 3 | $3.4 \times 10^7$ | 586 | 1,724 |
| 9 | $O_2$ | $N_2$ | $O_2$ | 35 | 120 | 155 | 3 | $3.3 \times 10^7$ | 529 | 1,603 |
| Initial cellular suspension | | | | — | — | — | 100 | $1.3 \times 10^9$ | 500 | 38 |

* $4.0 \times 10^{-6}$ M $FeSO_4$.

## DISCUSSION

The biological consequences of X-ray and ultraviolet irradiation and of treatment with certain chemical mutagens can be modified greatly by various pre- or posttreatment procedures. The lethal effect of X-rays for several strains of *E. coli*, including strain Sd-4, can be decreased by irradiating in the presence of nitrogen or in other ways reducing the concentration of oxygen present during exposure (Anderson, 1951; Hollaender *et al.*, 1951; Thompson *et al.*, 1951; Stapleton *et al.*, 1952). Reduction of the lethal action of ultraviolet radiation for *E. coli* has been achieved by pretreatment with sodium cyanide, carbon monoxide (Mefferd and Matney, 1952), or pyruvate (Thompson *et al.*, 1951). In the case of pyruvate, however, and also tryptophan, cysteine, thioglycolate, and glutathione, apparent protection has been found to result from physical absorption by the chemical during ultraviolet irradiation (Whitehead, 1952).

Application of visible light (Kelner, 1949) or of hydrostatic pressure (McElroy, 1952) after ultraviolet irradiation of cells reduces the lethal effects and produces partial reversal of mutagenic effects. Such evidence has suggested to a number of workers (for example, Kelner, 1950; Thompson *et al.*, 1951; McElroy, 1952) that some intermediate stage or latent period intervenes between absorption of radiant energy and occurrence of the biological events. The duration of such a stage would be conditioned by the physiological state of the cell as influenced by a number of factors, such as temperature, presence of sources of energy or nutrients, or toxic compounds. Supplementary treatment designed to modify the consequences of irradiation would have to be applied during this latent period to be effective.

In assessing the primary lethal effects produced in bacteria by a physical or chemical agent, and subsequent reversal of these effects, it is necessary to recognize also the influence of secondary physiological factors. After exposure, cells are more sensitive than normal to deleterious environmental conditions. Treated cells must be plated promptly on adequate media in order that the counts of survivors and mutants will reflect the actual state of the population immediately after exposure. Lethal consequences that may be associated with harsh posttreatment manipulations should be recognized as such. Conditions that merely prevent these secondary lethal consequences must be clearly distinguished from those that elicit progressive reduction of the agent's primary lethal effects, which alone represents genuine reversal or recovery.

In *E. coli*, strain Sd-4, the effects of exposure to $Fe^{++}$ at 1 C could be reversed partly by bubbling vigorously the reaction mixture with nitrogen and changing the exposure temperature to 37 C. That this recovery represents a genuine reversal of lethal effect was attested by the correlation between degree of recovery and duration of supplementary treatment. Successive samples of the reaction mixture over a period of nearly two hours revealed progressive increase in numbers of survivors. In speculating about the nature of the recovery process, it seems significant that the physiological condition of cells exposed to $Fe^{++}$ at 37 C in the presence of nitrogen was fairly similar to that of untreated controls in respect to synthetic activities, duration of the initial stationary phase, and fraction of survivors. Supplementary treatment under these conditions ($N_2$ at 37 C) may improve the metabolic state of cells that were exposed to $Fe^{++}$ at 1 C and, thus, increase the proportion of cells in which viability is retained because reactions initiated by $Fe^{++}$ at 1 C fail to proceed to completion. Recovery was not produced when the entire secondary exposure at 37 C was carried out in oxygen; however, the existence of an aerobic state during initial 1 C exposure to $Fe^{++}$ did not destroy the capacity for recovery of a portion of the population which then was exposed to supplementary treatment in nitrogen at 37 C.

The mutagenic effect of $Fe^{++}$, which was expressed as a great increase or "burst" of streptomycin-nondependent revertants when the 1-to-37 C sequence of exposure took place in air or in oxygen, was reduced strikingly when the exposure was carried out in nitrogen. The "burst" effect was regained, however, when the flow of nitrogen was replaced with oxygen during a period as brief as 5 minutes, corresponding to the time at which the 1-to-37 C change took place. This suggests that some degree of aerobiasis is required for the "burst" effect on reversion frequency.


## SUMMARY

*Escherichia coli*, strain B/Sd-4, was employed in studies of the influence of variations in oxygen tension and exposure temperature on the effects of $Fe^{++}$. Whether the $Fe^{++}$-bacteria mixtures are exposed in the presence of nitrogen or of oxygen, treatment at 1 C produces a far greater lethal effect and a higher frequency of reversion from streptomycin dependence to nondependence than treatment at 37 C. These effects of $Fe^{++}$ treatment at 1 C, which are greatly increased by extending the treatment to include a secondary period of exposure at 37 C in the presence of oxygen, are decreased when the 1-to-37 C sequence of treatment is carried out in the presence of nitrogen. The fraction of survivors at the end of this supplementary exposure in nitrogen at 37 C is actually considerably higher than at the end of the initial exposure to $Fe^{++}$ at 1 C. This represents recovery of part of the treated population.


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