EFFECT OF OZONE ON SURVIVAL AND PERMEABILITY OF *ESCHERICHIA COLI*

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ABSTRACT

SCOTT, D. B. McNAIR (University of Pennsylvania, Philadelphia) AND E. C. LESHER. Effect of ozone on survival and permeability of *Escherichia coli*. J. Bacteriol. **85**:567–576. 1963.—*Escherichia coli* cultures in the logarithmic phase or resting were treated with various concentrations of ozone in saline solution. Approximately 2×10^7 molecules of ozone per bacterium killed 50% of the cells. Ozone caused leakage of cell content into the medium, and lysis of some cells. Low concentrations of ozone did not react with the glutathione within the cells, although reaction with glutathione in solution was immediate and stoichiometric. The effect on nucleic acid within the cells was to change the solubility and to cause the release of ultraviolet-absorbing material into the medium. Ozone attacked the ring structure of the base or the carbohydrate only when the substance was in the medium. Nucleic acids released into the medium were reabsorbed by cells which were not lysed. Viable cells resumed growth immediately, and grew at rates determined by the nutrients either added to the medium or which resulted from leakage and lysis of nonviable cells. It is postulated that the primary attack of ozone was on the cell wall or membrane of the bacteria, probably by reaction with the double bonds of lipids, and that leakage or lysis of the cells depended on the extent of that reaction.

Ozone is formed by electric discharge through oxygen or air. It is also formed by ultraviolet (UV) radiation at 2,000 to 2,100 A, especially in the higher atmosphere (40 km or 15 to 30 miles). The concentration at the surface of the earth is about 10^{-8} (parts by volume). Ozone absorbs UV at 2,537 A and is decomposed. The deleterious effect of ozone on life is advantageous to man; ozone kills bacteria and is used for this purpose in sewage-disposal plants and in preservation of meat during the tenderizing process, etc. In this latter instance, a part of the effect of UV lamps in sterilizing is considered to be due to the ozone (0.1 ppm) produced by the wavelengths of 1,750 to 2,000 or 2,100 A (Nagy, 1959). The concentration of ozone which kills bacteria has been variously reported to be 0.04 to 0.1 ppm (volume), whereas the toxicity for small animals is 3 to 12 ppm (Stockinger, 1959). Humans experience headache, and dryness and irritation of the throat, respiratory passages, and eyes at 0.1 ppm. At that concentration, the odor of ozone is disagreeable, and most people can detect the odor at 0.02 to 0.04 ppm.

Davis (1959) has shown that ozone is also mutagenic, in fact, one of the most potent mutagens known. Perhaps some of the mutagenic effects of UV irradiation may be attributed to the ozone produced by the shorter wavelengths. The exact mode of action of ozone in killing bacteria has not been determined. Ozone is such a strong oxidizing agent that it reacts with many substances of biochemical importance. It has been postulated that the primary bactericidal reaction is the oxidation of SH^- to S–S (Barron, 1954).

It was to test this hypothesis that our experiments were undertaken. We found that, on the contrary, the SH^- concentration of bacteria was not decreased until it leaked out or the cells were lysed. The attack of the ozone seemed to be at the cell surface, with alteration of the permeability of the membrane. It is postulated that the primary attack of ozone is on the double bonds of unsaturated lipids in the cell membrane.

MATERIALS AND METHODS

Ozone was produced by a Welsbach model T-23 laboratory ozonator at a voltage of 100 and 8-lb dry oxygen pressure, with a flow rate of 0.2 ft^3 per min. The ozone was passed directly through the bacterial culture in some experiments. In the majority of experiments, the ozone was passed

through saline solutions for a chosen time (from 20 sec to 5 min). Saturation time was between 2 and 5 min. The solution was then added immediately to suspensions of bacteria in saline and alternately to solutions of alkaline 10% KI. For treatment of solutions of pure chemicals, such as the nucleic acids, nucleotides, etc., the ozone was bubbled through doubly distilled water surrounded by an ice bath. To obtain stable solutions of ozone and reproducible results for the concentration of ozone in the solutions, it was necessary to use glass-distilled water and to rinse all the glassware well with the same. It was found that the solutions of ozone in glass-stoppered cylinders were very stable at 0 to 4 C, whereas there was a slow loss of ozone at room temperature. The bacterial cultures were treated with ozone solutions at room temperature to avoid cold shock to the bacteria. The uncertainty of ozone concentration (calculated per 10^9 bacteria) due to this factor and to the variable loss of material which could react with ozone, from the bacteria into the suspending media, was minimized by working as fast as possible and reproducing the same conditions in each experiment.

Escherichia coli B was cultivated in salts-glucose medium in an aeration tower at 36 C, as described previously (Scott and Chu, 1958). Turbidity of the cultures was determined in a Klett-Summerson photometer. The total bacteria were counted microscopically in a Petroff-Hauser bacterial counter. The viable bacteria were determined by the number of colonies which grew on plates of nutrient agar (overnight), or salts-glucose agar (72 hr), after spreading of suitable dilutions of the cultures, before and after treatment with ozone.

E. coli B (0.01 ml of a weekly culture taken from a slant) was inoculated into 200 ml of salts medium containing glucose (2 mg/ml). After incubation overnight in a water bath at 37 C with aeration, the turbidity with a 420-mμ filter was approximately 300 Klett units, growth being limited by exhaustion of glucose. The resting culture was centrifuged, and the cells were washed and resuspended in 6 ml of saline; 1 ml of the suspension was added to each of four glass-stoppered centrifuge tubes containing volumes of saline calculated to give a final volume of 25 ml. Three different volumes of ozone in saline solution were added alternately to tubes containing culture and to tubes containing the same volume of KI in saline. Samples of the treated cultures were then plated on salts-glucose and nutrient agar plates in several dilutions and incubated. To obtain growing cultures, a portion of the resting culture was inoculated into an aeration tower containing salts-glucose medium to give a turbidity of 50 and a volume of 150 ml. After 2 hr of aeration at 37 C, the cells were harvested and treated with ozone in the same way as the resting cells.

The ozone concentration was determined by acidification of the alkaline KI solution with 3 M acetic acid and measurement of the optical absorbance at 352 mμ, according to the method described by Davis (1959).

For determination of nucleic acids in the bacteria, 1-, 2-, or 5-ml samples of the cultures were centrifuged. The bacterial pellets were treated as follows. The low-molecular-weight nucleotides and nucleosides were extracted by treatment with 1 M NaCl or with cold 2% perchloric acid. Approximately one-half of the ribonucleic acid (RNA) was extracted with cold 5% perchloric acid overnight. The remaining RNA and the deoxyribonucleic acid (DNA) were extracted with hot (90 C) 5% perchloric acid for 5 min. In some experiments, the total nucleic acid was extracted with hot 5% perchloric acid. The nucleic acid contents were determined in these fractions, in whole unfractionated cells, and in the residues after extraction with cold or hot perchloric acid.

DNA, deoxynucleotides, and deoxynucleosides were determined by the reaction of the deoxyribose attached to the purine moiety with diphenylamine, according to the procedure of Burton (1956). Similarly, RNA, the ribonucleotides, and ribonucleosides were determined by reaction of the purine-bound ribose with orcinol, according to the procedure of Miller, Golder, and Miller (1951). The spectra of the solutions of nucleic acids, nucleotides, nucleosides, bases, and of the media, before and after treatment with ozone, were graphed from measurements in a Beckman model DU spectrophotometer.

Glucose was determined by the glucose oxidase method with a kit supplied by Worthington Biochemical Corp., Freehold, N.J.

The glutathione content of the bacteria was determined by amperometric titration of extracts obtained by treatment with cold (−15 C) 70% ethanol. The titration procedure was a modifi-

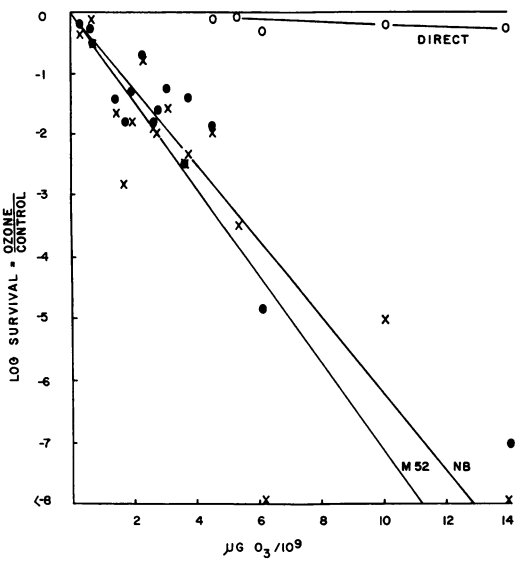

FIG. 1. *Survival of logarithmic-phase Escherichia coli after treatment with ozone.*

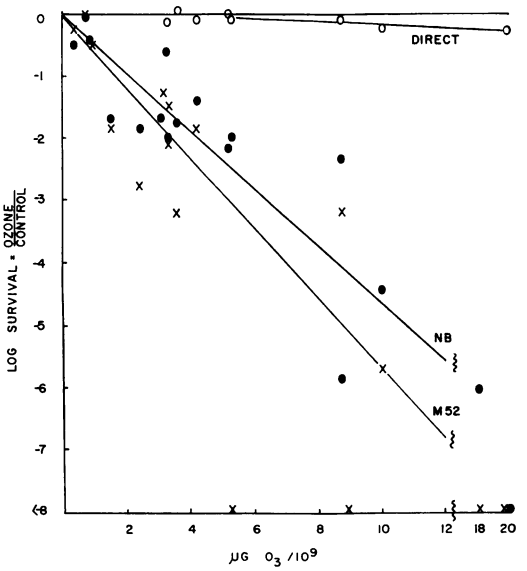

FIG. 2. *Survival of resting Escherichia coli after treatment with ozone.*

cation of that described by Benesch, Lardy, and Benesch (1955).

The ethanol extracts were also treated with *N*-methylmaleimide and submitted to chromatography, as described by Benesch et al. (1956).

RESULTS

Survival. The effect of treatment with solutions of ozone is shown in Fig. 1 for growing cultures and in Fig. 2 for resting cultures. In both figures, the lines have been drawn through the median values of the individual slopes; the medians differed very little from the mean values. These two values were about 20% higher than the calculated regression coefficients which weighted the data in favor of the higher concentrations of ozone. Lower bacterial counts suffered from greater variability. The regression calculations also gave appreciable values of "*a*" in the term $y = a + bx$. For example, the largest a value was for growing cultures plated on synthetic medium, i.e., $y = -0.47 + (-0.55)x$. The a values may result from the counts of untreated cultures being lower on the synthetic medium than on nutrient broth, or from the obvious shifts in the effects of ozone with increasing concentrations. These effects are shown in Table 1, in which are listed the survival rates and the ratios of growth on salts-glucose and nutrient broth plates, and a calculation of excess "depleted cells." This term indicates the percentage of bacteria which would grow on nutrient broth and not on synthetic medium, corrected for this same factor shown by untreated cells. There was a shift in survival to less than 10% at about 1 μg of $O_3/10^9$ bacteria with both resting and growing cultures. There was another shift to less than 1% survival at about 5 μg of $O_3/10^9$ bacteria.

The concentration of ozone producing 50% or more "excess depleted cells" among the survivors was approximately 1.5 $\mu g/10^9$ with growing cultures and between 2 and 4 $\mu g/10^9$ with resting cultures. With survival rates of less than 1%, most of the survivors seemed to be "depleted."

Microscopic inspection and counting of cultures treated with solutions of ozone indicated that there had been only slight immediate lysis of cells, less than 50% even at the higher concentrations. After treatment with concentrations greater than 1 μg/ml, the bacteria were no longer observed to be motile. After ozone was bubbled through the cultures, no bacteria were intact and most had lysed, leaving "ghosts." No ozone was detectable in the media after treatment of cultures either by ozone in solution or after bubbling was stopped.

Effect on glutathione. By chromatography, glutathione was found to be the only sulfhydryl-

TABLE 1. *Survival and depletion after treatment of growing Escherichia coli with ozone*

Expt no.	O_3	Untreated M52*/NB × 100	Ozone			
			Survival		M52/NB × 100	Excess "depleted cells"
			On NB	On M52		
	μg/10⁹	%	%	%	%	%
V	0.25	80	72	40	56	24
IV	0.59	83	61	72	120	0
III	0.65	94	40	34	85	9
III	1.35	94	4.2	2.5	60	34
V	1.66	80	1.9	0.45	24	56
VII	1.9		6.8	3.0	45	40†
VIII	1.9		6.0	1.9	32	53†
VI	2.0	109	1.9	1.6	87	13
IV	2.7	83	3.1	1.0	33	50
IX	3.0		6.7	3.5	52	33†
III	3.6	94	0.39	0.42	110	0
V	3.7	80	4.9	0.45	9	71
IV	4.5	83	1.6	1.0	67	16
II	5.3	85		0.04		
I	6.1		0.0016	0		100†
II	10.3	85		0.001		
I	14.0		0.00001	0		100†

* M52, salts + glucose medium; NB, nutrient broth.

† Estimated.

TABLE 2. *Survival and depletion after treatment of resting Escherichia coli with ozone*

Expt no.	O_3	Untreated M52*/NB × 100	Ozone			
			Survival		M52/NB × 100	Excess "depleted cells"
			On NB	On M52		
	μg/10⁹	%	%	%	%	%
V	0.36	80	69	44	63	17
IV	0.67	48	90	67	74	0
III	0.86	110	36	36	100	0
III	1.7	110	2.0	2.0	100	0
V	2.4	80	1.3	0.13	10	70
IV	3.1	48	2.0	2.7	135	0
VI	3.25	75	1.05	0.7	67	8
I	3.3	60	24	1.9	8	62
III	3.6	110	1.8	0.07	3.8	96
II	4.2	32	5.0	0.5	10	22
V	5.2	80	0.64			
IV	5.3	48	1.0	0		100
VI	8.7	75	0.07	0.009	13	62
I	8.7	60	0.00014	0		100
II	10.0	32	0.0037	0.00007	1.9	30
II	18.0	32	0.0001	0		100
I	20.0	60	0	0		0

* M52, salts + glucose medium; NB, nutrient broth.

containing compound in detectable amounts in the 70% alcohol extract of the bacteria. The concentration determined by titration of the alcohol extracts of untreated bacteria was variable, according to the stage in the growth cycle of the culture. With duplicate samples, the mean variation from the mean was 8% in the experiments reported here. The content of glutathione of untreated *E. coli* was found to vary widely (from 2 to 14 mμmoles/10^9 cells). If growing cultures of *E. coli* were held at 6 C in synthetic medium without glucose for 1 hr, as is done to produce synchrony of division, the glutathione contents were 7 to 10 mμmoles/10^9. Warming to 37 C might or might not lower the values. Addition of glucose caused the values to drop to 2 to 4 mμmoles, with a slow increase to 5 or 6 mμmoles/10^9 before doubling of the bacterial count at 60 min. Addition of nitrogen mustard (Scott, Lesher, and Rosenbaum, *unpublished data*) or *N*-methylmaleimide (Stern, 1960) in amounts which prevented or delayed division, without effect on turbidity, caused increases of glutathione content to as much as 10 to 12 mμmoles/10^9 bacteria.

There were no SH^- groups available to react with silver on the surface of whole untreated *E. coli*. Mixtures of solutions of glutathione and ozone resulted in a loss of titrable SH^- proportional to the ozone added. Table 2 lists the results of four sets of titrations of the alcohol extracts of bacteria, untreated or treated with ozone. After treatment with high concentrations of ozone, very little or no SH^- was detectable in the extracts. Lower ozone concentrations usually decreased the SH^- content but, at times, no change or even an increase was found in the SH^- of extracts of resting cultures, the viability of which might be less than 10%.

Effect on nucleic acid. When ozone was allowed to bubble through a bacterial suspension, the absorption (at 2,600 A) measurable in the supernatant fluid was increased (Fig. 3), with less absorption after 30 than after 10 sec. In the same experiment, suspensions of growing cells at half the count were treated with bubbling ozone for the same time; after 30 sec of ozone, the super-

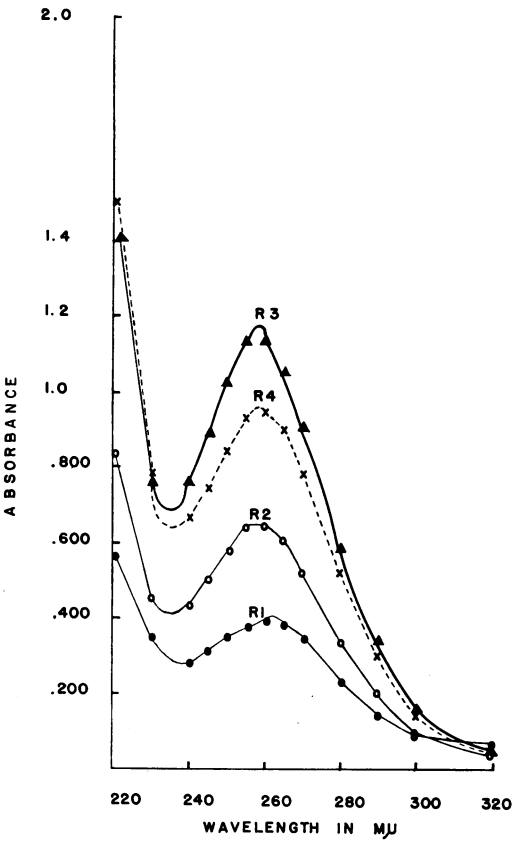

FIG. 3. *Effect of bubbled ozone on nucleic acids in the medium. Resting cells were treated by direct bubbling of ozone through a suspension of 1.7 × 10^9 cells/ml in 25 ml of saline for* R 1 = *control;* R 2 = *5 sec;* R 3 = *10 sec; and* R 4 = *30 sec. The cells and protein were sedimented by treatment with perchloric acid (2.5%) and centrifugation. Spectra were read on the supernatant fluid in a Beckman model DU spectrophotometer. The number of viable cells on nutrient agar were* R 1: *1.7 × 10^9;* R 2: *3.4 × 10^8;* R 3: *4.0 × 10^8;* R 4: *3.6 × 10^6. On salts-glucose,* R 1: *1.1 × 10^9;* R 2: *2.0 × 10^8;* R 3: *1.2 × 10^8; and* R 4: *not determined.*

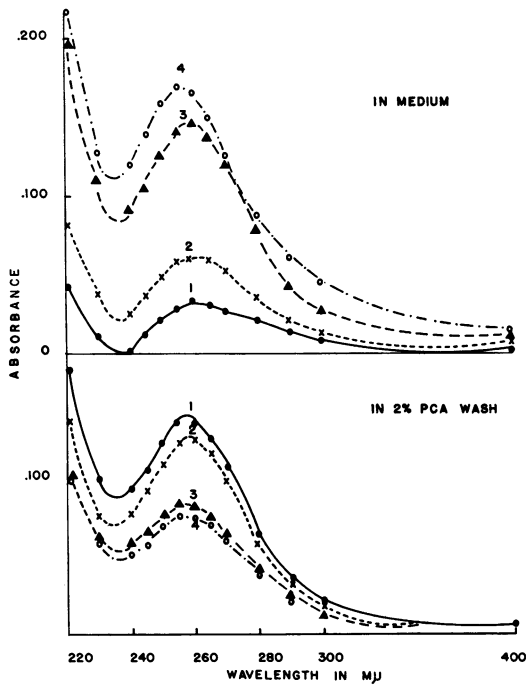

FIG. 4. *Effect of ozone solutions on nucleic acids. Cultures of growing Escherichia coli were harvested and suspended in saline with a final volume of 25 ml and a count of 7 × 10^8 cells/ml. The ozone solutions added were* 1: *none;* 2: *0.18 μg/ml;* 3: *1.27 μg/ml; and* 4: *2.5 μg/ml. Viable bacteria by plating on nutrient agar were* 1: *6.7 × 10^8;* 2: *4.8 × 10^8;* 3: *1.3 × 10^7; and* 4: *3.3 × 10^7. On salts-glucose agar,* 1: *5.5 × 10^8;* 2: *2.7 × 10^8;* 3: *3.0 × 10^6; and* 4: *3.0 × 10^6. Spectra were read on untreated media (pH 7.6) and on the two combined 1-ml washings with 2% perchloric acid of the pellet from 2 ml of culture.*

natant had no peak at 260 mμ, and after 10 sec the peak was lower than after 5 sec.

In a similar experiment, the protein released into the medium was 53 μg/ml from resting cells and 31 μg/ml from growing cells after passage of ozone for 30 sec.

After treatment with solutions of ozone, there was an increase in absorbance of the medium with as little as 0.18 μg of O_3/ml (3.75 × 10^{-6} M). With concentrations of 3 μg/ml (6 × 10^{-5} M) or higher, there was less absorbance than at lower concentrations. Concomitant with the increase of absorbance in the medium was a decrease of absorbance in the material removed by washing with 2% perchloric acid (Fig. 4). The release of material into the medium was usually greater by the growing cultures than by resting cultures. The medium and various fractions of the bacteria were analyzed for DNA and RNA by the sugar reactions with diphenylamine and orcinol, respectively. Figure 5 shows the increase in DNA in the medium and the decrease in the DNA determined in whole cells, in cold perchloric acid-extracted cells, and in the hot perchloric acid extracts, after treatment of resting cultures with increasing concentrations of ozone. The

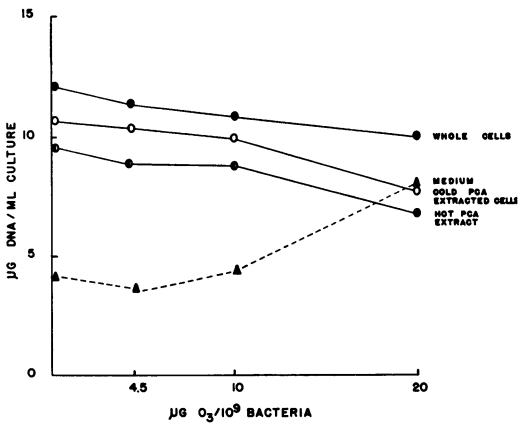

FIG. 5. *Effect of ozone on deoxyribonucleic acid (DNA) of resting cultures. To resting-cell suspensions of 3.7 × 10^8 bacteria per ml were added ozone solutions to give concentrations of 1.7, 3.7, and 7.4 μg/ml of ozone. DNA was determined by the diphenylamine reaction in the medium, the whole cell, the cells extracted with cold 5% perchloric acid, and the hot 5% perchloric acid extract.*

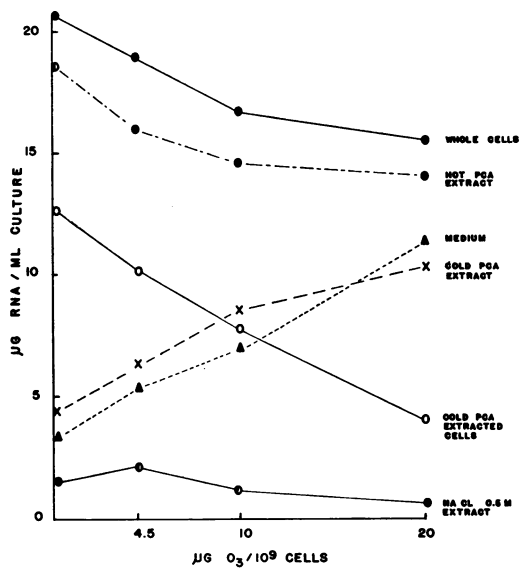

FIG. 6. *Effect of ozone on ribonucleic acid (RNA) of resting cultures. Samples of the same suspensions of bacteria as shown in Fig. 5 were analyzed for RNA by the orcinol method. Solutions are as for Fig. 5 and, in addition, RNA was determined in the cold 5% perchloric acid extract, and the 0.5 M NaCl extract.*

pattern is similar after ozone treatment of growing cultures.

The effect of the ozone solution on the RNA fractions appears in Fig. 6. Orcinol-reacting material was increased in the medium and also in the fraction extractable with cold 5% perchloric acid. The latter seemed to be derived from the solubilization of RNA which is not usually extracted by cold perchloric acid and is shown as the residual RNA in cold-extracted cells or as that extracted with hot perchloric acid after cold extraction. Growing cells contained at least twice as much RNA as resting cells, distributed in about the same proportions in the fractions. With ozone treatment, there was more RNA lost into the medium and less increase in that extractable with cold perchloric acid, while the decrease in the residual RNA was approximately the same in amount, although less, proportionately, than in resting cells.

It is evident that more than 10 μg of ozone/10^9 bacteria were necessary to produce a loss of DNA into the medium, whereas 4.5 μg of ozone caused a significant increase in RNA in the medium and a change in solubility in 5% perchloric acid of the RNA inside the cells.

Recovery after ozone treatment. Cultures treated with about 2 μg of $O_3/10^9$ bacteria were diluted into various media, and the resumption of growth was investigated by determination of direct counts, turbidity, the uptake of nutrients from the medium, and the content of nucleic acids in the hot perchloric acid extract. Figure 7 shows the initial loss and then increase of turbidity by ozone-treated cultures diluted into nutrient broth, into salts-glucose medium, into salts-glucose medium fortified with casein hydrolysate, and salts medium with casein hydrolysate alone. Also shown are the glucose uptake by the two cultures containing glucose, and the uptake of ribose-containing material by the culture in nutrient broth. After ozone treatment, 6.8% of cells were viable on nutrient broth plates and 3% of cells were viable on salts-glucose plates. After 25 min in all three media, by direct count 35% of the cells had lysed. The loss of RNA from the cells at that time was 26, 43, and 51% in glucose + casein hydrolysate, glucose, and casein hydrolysate, respectively. These results would indicate that in the casein hydrolysate medium some cells which had not lysed had lost RNA, whereas in glucose + casein hydrolysate some cells had taken up from the medium some of the RNA released from lysed cells.

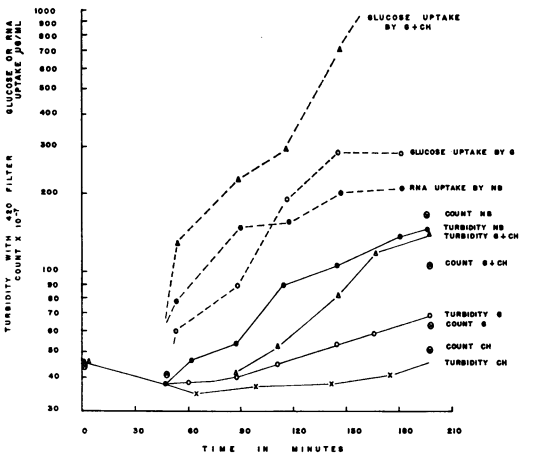

FIG. 7. *Growth after ozone treatment. Escherichia coli were harvested after 2 hr of growth in salts-glucose medium and suspended in 40 ml of saline mixed with 60 ml of saline through which ozone had bubbled for 1 min. Cell count was 1.76 × 10⁹ bacteria/ml, and ozone concentration was 6 μg/ml; 25 ml of the treated cultures were added to each of four towers with a total volume of 100 ml. Tower 1 contained 24 ml of saline, 50 ml of double-strength M52, and 2 mg/ml of glucose* (○, G). *Tower 2 contained 23 ml of saline, 50 ml of double-strength M52, glucose (2 mg/ml), and 1 mg/ml of casein hydrolysate* (△, G + CH). *Tower 3 contained 25 ml of saline and 50 ml of double-strength nutrient broth (Difco);* (●, NB). *Tower 4 contained 24 ml of saline, 50 ml of double-strength M52, and 1 mg/ml of casein hydrolysate* (×, CH). *The towers were incubated with aeration at 37 C. Count is the direct microscopic cell count.*

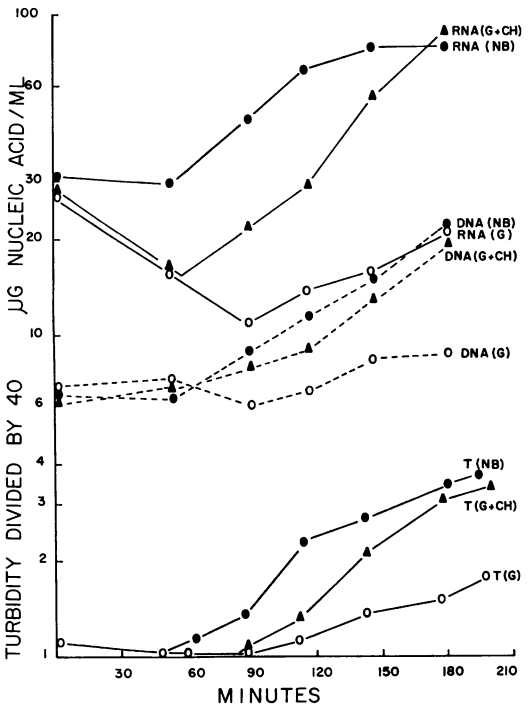

FIG. 8. *Nucleic acids during growth after ozone. Nucleic acids were determined in hot perchloric acid extract of samples of the cultures described in Fig. 7.* T *represents turbidity in Klett units with a 420-mμ filter.*

For a similar experiment, Fig. 8 shows the increase of RNA and DNA extracted with hot perchloric acid. It would seem that, when the bacteria took up nutrient from the medium, they started to grow again; the lag was shorter and growth faster the richer the nutrient medium. There was loss of RNA from the bacteria until the time when some were able to take up nutrient and start growing. Approximate values for nucleic acids, nucleotides, and nucleosides in the cell-free media, obtained by measurement of the absorbance at 255 to 300 mμ at pH 2, indicated increases of 10 and 12 μg/ml during the first 40 min of culture in salts + glucose and salts + glucose + casein hydrolysate, respectively. During the same time, the sedimented bacteria lost 11 and 7 μg of RNA/ml, respectively. From 98 to 177 min, while the RNA in the bacteria was increasing 20 μg/ml in the culture with glucose alone, the absorbance of the medium decreased equivalent to approximately 3.5 μg/ml of nucleic acid. Between 67 and 100 min, the faster-growing culture in salts medium containing glucose + casein hydrolysate showed an increase of 16 μg of RNA/ml in the bacteria and a decrease of 5.6 μg of nucleic acid/ml in the medium. Thus, the viable bacteria both removed UV-absorbing material from the medium and synthesized RNA within the cells.

In the upper part of Fig. 9 are plotted the direct counts of bacteria at times during the experiment. However, from the results of plating, only 2% of the culture was able to grow on salts-glucose and only 6% on nutrient broth. In the lower part, the numbers of dead bacteria in the culture were subtracted from the direct counts, and the numbers of viable cells were plotted. It can be seen that, in 2 hr, four generations of cells in nutrient broth produced a viable population equal to that before ozone treatment; the

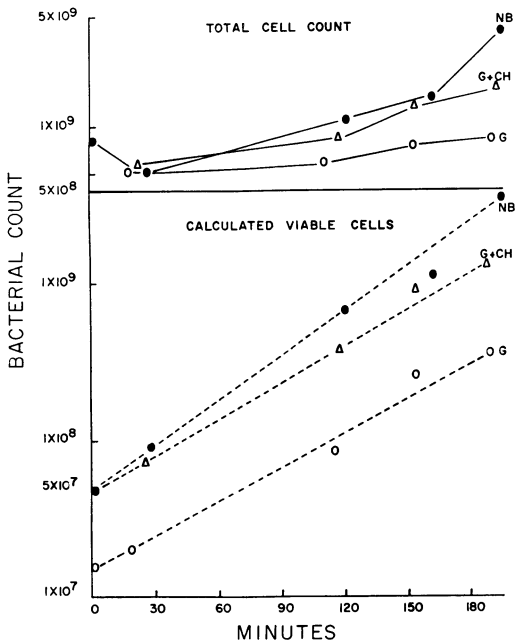

FIG. 9. *Total and viable cells during growth after ozone treatment. The cultures were from an experiment set up in the same way as Fig. 7. The ozone concentration was 1.9 μg/10⁹ bacteria. The treated culture was plated immediately, and the viable count graphed at 0 time. The nonviable cell count was subtracted from the direct counts (upper graph) at the indicated times to give the plotted calculated viable counts in the lower graph.*

doubling time was 30 min. On salts-glucose, six generations with a doubling time of approximately 45 min would have reached the same population at about 270 min if the experiment had been continued. The usual division time of *E. coli* in salts-glucose under the conditions in our laboratory is 55 to 60 min. With addition of casein hydrolysate to the salts-glucose, the doubling time was reduced to 40 min, and the population was replaced in approximately 160 min. It must be concluded that the early period did not represent a lag phase in the usual sense for all the bacteria in the culture. During this time, the nonviable bacteria (more than 90%) were losing ribonucleotides or ribonucleosides and protein into the medium and probably other cell substance which we did not measure. Approximately 50% were lysing completely, while the viable cells started growth immediately at rates determined by the added nutrients and the decomposition products of the nonviable bacteria in the medium.

Mutagenesis. Since after ozone treatment at concentrations of 2 to 5 μg of $O_3/10^9$ bacteria approximately twice as many bacteria were viable in nutrient broth as in salts-glucose medium, it might be suspected that half the viable bacteria were "mutants." Because of the effect of casein hydrolysate noted above, several attempts were made to isolate amino acid-requiring mutants. Several cultures were obtained, all of which required methionine which was not replaceable by vitamin B_{12}.

Effect of ozone on nucleic acids. We also investigated the effect of ozone on purified nucleic acids and on the nucleotides, nucleosides, and purine and pyrimidine bases. The effects at low concentrations were attacks on the pyrimidine rings and loosening of the bond between the pyrimidine base and the sugar moiety, so that the latter could react with diphenylamine or orcinol in the usual analytic procedures. At higher concentrations, our results were similar to those of Christensen and Giese (1954), in that the pyrimidine and purine rings were broken and the sugars decomposed. The results of these studies will be reported elsewhere in detail.

DISCUSSION

It seemed evident that, in the treated cultures, the ozone attacked the primary structure of nucleic acids or their decomposition products only after they had been released into the medium by leakage or lysis. The effect of ozone on the nucleic acids within the cells may have been indirectly on the states of aggregation of the nucleoproteins, and not directly on the nucleic acids themselves. That the ozone did not seem to penetrate the cells is indicated also by the inability of even fairly high concentrations of ozone (up to 40 times the glutathione equivalents) to oxidize all the SH^- of glutathione contained within the cell. The loss of protein and nucleic acids by the ozone-treated cells implies that the primary locus of activity of ozone was the bacterial-cell surface, as suggested by Christensen and Giese (1954). Since we have not been able to detect any SH^- groups by amperometric titration of whole bacteria, we conclude that there are no SH^- groups available on the cell surface to react with ozone.

Other substances with which ozone reacts

rapidly and completely include unsaturated fats. Hann (1950), quoting Harries (1910) and others, reported that ozone reacted with oleic acid rapidly even at temperatures as low as −78 C to produce ozonides, and that hydrolysis or reduction of the ozonides broke the C–C bond, producing two fatty acids of nine carbons each.

According to Salton (1960), 15% of the dry weight of *E. coli* is cell wall. Of the cell wall, 23% is lipid and 13% is phospholipid. Phospholipid makes up 20% of cytoplasmic membrane (Mohan, *personal communication*).

An iodine number for this lipid has not been found in a search of the literature. Grylls (1961) gives the iodine number for yeast lipid as 130. Assuming that *E. coli* lipid has approximately the same iodine number, we may calculate that the lipids in the cell wall of one bacterium will have 10^8 double bonds.

At a concentration of ozone which kills 50% of a culture, the calculated number of molecules of ozone per bacterium is approximately 2×10^7; for 1% survival, the number is approximately 4×10^7.

Thus, the number of lipid double bonds in the cell wall may be of the same order of magnitude as the number of molecules of ozone which kill one *E. coli* bacterium. It is also of the same order as the number of molecules of iodine which are removed from solution by one bacterium (approximately 7×10^7; Scott and Lesher, *unpublished data*). It is also of interest that this is the same order of magnitude as the number of molecules of nitrogen mustard (bischlorethylaminoethane) per bacterium which will kill 50% of a culture. So it may be postulated that the primary attack of ozone, of iodine, and perhaps of nitrogen mustard is on the double bonds of the fatty acids in the cell wall and membrane of the bacterium.

Nathan (1961) reported experiments on induction of oxalacetate decarboxylase synthesis in *Lactobacillus plantarum* by malic acid in the presence but not in the absence of chlorpromazine. It was suggested that chlorpromazine altered the cell membrane so that it was permeable to malic acid, at drug concentrations which did not affect viability. Nathan and Friedman (1962) indicated that the site of action of chlorpromazine may be a lipid.

Whether the primary site of attack of ozone on the cell membrane of *E. coli* was on the unsaturated lipid or not, the end result was increased permeability. Since the ozone caused leakage of the contents of cells that were not lysed, it is probable that some of the cells were so depleted that they could not resume growth on salts and glucose alone but were able to grow on media that supplied more complicated organic materials, which might act as "pump primers." This might explain the observation that the higher the concentration of ozone and the fewer survivors there were, the greater was the proportion of them which grew on the enriched medium.

Davis (1959) found very high rates of mutation from streptomycin dependence to nondependence, but slight increase in frequencies of phage resistance, after exposure to ozone. It is difficult to conceive of the mutagenesis of ozone on the basis of a primary reaction with lipid. However, ozone was found to react with nucleic acids released into the medium, especially with the pyrimidine bases (thymine being more easily attacked than cytosine and uracil). If these changed bases were then assimilated by the still-viable bacteria and built into the nucleic acids, we might expect to find mutants among the offspring of the survivors. We were able to pick out only a few mutants, all methionine-requiring, when we tested for amino acid mutants. The method for isolating mutants involved centrifugation and washing of the bacteria before they were suspended in salts-glucose medium containing penicillin. Thus, the products of leakage and lysis, including those changed by ozone, may have been removed before the viable cells had a chance to absorb them. In other attempts to isolate mutants, an ozone-treated culture was allowed to grow in nutrient broth before treatment with penicillin. All colonies picked as mutants grew on minimal medium when plated again, so no true mutants were obtained.

In the work reported here, the nucleic acids in the medium were not isolated to determine whether they were RNA, DNA, mono- or polynucleotides, or nucleosides. However, little or no DNA was lost from cells treated with less than 10 μg of ozone per 10^9 bacteria, so the nucleic acid lost to or taken up from the medium was mainly RNA.

That chemically altered RNA may be mutagenic for a RNA-containing virus was shown by Gierer and Mundry (1958). They produced

mutants of tobacco mosaic virus by treatment of its separated RNA with sodium nitrite. They found that, if the RNA was treated for a time which reduced the incidence of infection to 50%, 19.5% of the infectious units were mutants of one kind, which produced necrotic rather than chlorotic lesions on Java tobacco leaves. When tested on Samsun tobacco, 33 of 60 (55%) of the progeny virus isolated produced a variety of symptoms different from the untreated TMW virus or RNA, whereas 20 of the 60 were no longer viable (Mundry and Gierer, 1958).

Litman and Ephrussi-Taylor (1959) reported that isolated DNA of pneumococci, which was treated with nitrous acid, produced mutants for drug resistance at 10% of the rate of mutation produced by transforming DNA. Ultraviolet irradiation of the DNA produced only inactivation and no mutants.

It may be that the cells which we at first isolated as mutants were temporarily modified by assimilation of altered RNA, whereas Davis's mutants for streptomycin nondependence might have resulted from uptake of DNA or deoxyribosides altered by ozone. Certainly, further work is required to elucidate the mutagenic action of ozone.

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LITERATURE CITED

BARRON, E. S. G. 1954. The role of free radicals and oxygen in reactions produced by ionizing radiations. Radiation. Res. **1**:109–124.

BENESCH, R., R. E. BENESCH, M. GUTCHO, AND L. LAUFER. 1956. New color test for thiols and thiolesters. Science **123**:981–982.

BENESCH, R. E., H. A. LARDY, AND R. BENESCH. 1955. The sulfhydryl groups of crystalline proteins. I. Some albumins, enzymes, and hemoglobins. J. Biol. Chem. **216**:663–676.

BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.

CHRISTENSEN, E., AND A. C. GIESE. 1954. Changes in absorption spectra of nucleic acids and their derivatives following exposure to ozone and ultraviolet irradiation. Arch. Biochem. Biophys. **51**:208.

DAVIS, I. 1959. The survival and mutability of *Escherichia coli* in aqueous solutions of ozone. Ph.D. thesis, University of Pennsylvania Medical School, Philadelphia.

GIERER, A., AND K. W. MUNDRY. 1958. Production of mutants of tobacco mosaic virus by chemical alteration of its ribonucleic acid *in vitro*. Nature **182**:1457–1458.

GRYLLS, F. S. M. 1961. The chemical composition of yeasts, p. 1050–1053. *In* E. Long [ed.], Biochemists handbook. D. Van Nostrand Co., Inc., Princeton, N. J.

HANN, V. A. 1950. "Tonnage ozone" for chemical processing. Chem. Industries (Philadelphia) **67**:386–389.

HARRIES, C. 1910. Über die Einwirkung des Ozones auf organische Verbindung. Ann. Chem. **374**:288–368.

LITMAN, R. M., AND H. EPHRUSSI-TAYLOR. 1959. Inactivation et mutation des facteurs génétique de l'acide désoxyribonucléique du pneumocoque par l'ultra violet et par l'acide nitreux. Compt. Rend. **249**:838–840.

MILLER, G. L., R. H. GOLDER, AND E. E. MILLER. 1951. Effect of varying proportions of components of Bial's color reagent. Anal. Chem. **23**:903–905.

MUNDRY, K. W., AND A. GIERER. 1958. Die Erzeugung von Mutationen des Tabakmosaikvirus durch chemische Behandlung seiner Nucleinsäure *in vitro*. Z. Vererbungslehre **89**:614–630.

NAGY, R. 1959. Application of ozone from sterilamp in control of mold, bacteria, and odors, p. 57–65. *In* Ozone chemistry and technology. American Chemical Society, Washington, D.C.

NATHAN, H. A. 1961. Alteration of permeability of *Lactobacillus plantarum* caused by chlorpromazine. Nature **192**:471–472.

NATHAN, H. A., AND W. FRIEDMAN. 1962. Chlorpromazine affects permeability of resting cells of *Tetrahymena pyriformis*. Science **135**:793–794.

SALTON, M. J. R. 1960. Microbial cell walls. John Wiley & Sons, Inc., New York.

SCOTT, D. B. MCN., AND E. CHU. 1958. Synchronized division of growing cultures of *Escherichia coli*. Exptl. Cell Res. **14**:166–174.

STERN, H. 1960. Biochemical sequences in mitosis, p. 135–165. *In* D. Rudnick [ed.], Developing cell systems and their control. 18th Growth Symposium, Ronald Press Co., New York.

STOCKINGER, H. E. 1959. Factors modifying toxicity of ozone, p. 360–369. *In* Ozone chemistry and technology. American Chemical Society, Washington, D.C.