A study of the action of oxygen, hydrogen dioxide, and ozone gas upon the growth of certain bacteria / by S.E. Finch.

P. C. 5

4.

A STUDY OF THE ACTION OF OXYGEN, HYDROGEN DIOXIDE, AND OZONE GAS UPON THE GROWTH OF CERTAIN BACTERIA.

BY

S. E. FINCH, M.D.,

NEW YORK.

Reprinted from the

New York Medical Journal

INCORPORATING THE

Philadelphia Medical Journal and The Medical News.

November 29, 1913.

Reprinted from the New York Medical Journal for November 29, 1913.

A STUDY OF THE ACTION OF OXYGEN, HYDROGEN DIOXIDE, AND OZONE GAS UPON THE GROWTH OF CERTAIN BACTERIA.*

By S. E. Finch, M. D.,
New York.

The physiological and the mechanical action of oxygen gas when introduced within the peritoneum of normal animals and of man, was found by Bainbridge (1) and by Meeker (2), "to justify the assumption that the intraabdominal use of oxygen is entitled to a place in surgical therapy." The beneficial effects obtained by the use of ninety-five per cent. oxygen intraabdominally in the human subject following severe laparotomies, (3) in particular, the favorable progress of certain cases with associated local or general intraperitoneal infection in which it was used, raised the question as to whether or not an antiseptic action is included in the therapeutic action of oxygen. At the suggestion of Dr. W. Seaman Bainbridge, a study of the action of oxygen upon the growth of certain microorganisms was undertaken in the laboratory of the New York Skin and Cancer Hospital. The results obtained with oxygen led to a study of the bactericidal action of hydrogen peroxide and of ozone. The microorganisms used were virulent strains of aurococcus, *Streptococcus brevis, Bacillus coli,* and one strain of the *Bacillus tuberculosis* (human type).

TECHNIC.

The culture media used for growing these bacteria were meat infusion, glycerin, and serum agar, plain and glycerin bouillon. When it was desired

*From the Research Laboratory of the New York Skin and Cancer Hospital.

to grow them in an atmosphere of oxygen, the organisms were grown in litre and half litre flasks on solid media. For use with a continuous current, they were grown in test tubes and in flasks in fluid media. Where suspensions of the bacteria were required, several slants of one strain of an organism were grown for twenty-four hours at 37° C. and the growth washed off from each slant with two cubic centimetres of sterile water and transferred by a sterile pipette to a large sterile tube; the bacteria in this tube were thoroughly emulsified, and two cubic centimetres of this suspension regarded as representing one culture in conducting the experiments. To keep the different strains of bacteria that were employed, virulent, they were passed through mice or guinea pigs at frequent intervals.

The oxygen gas used represented ninety-five per cent. of pure oxygen. The oxygen was employed at 22° C. when used as a continuous current and for replacing the air in flasks. For the latter purpose oxygen was led from its tank through sterile apparatus, introduced just above the surface of the medium in the bottom of a flask and allowed to flow until from five to six litres had thus entered the vessel at the lowest point possible, with free exit for the gases at the top. In each instance before connection with the oxygen tank was severed, screw clamps, securely fastened on the heavy rubber tubing leading to and from the vessel, rendered leakage impossible.

The hydrogen dioxide (U. S. P. three per cent.) was obtained from two different sources of manufacture, and for each group of experiments was taken from freshly opened bottles. All apparatus for this work was sterilized previous to use. Twenty-four hour fluid cultures, and suspensions of twenty-four hour slant growths, as described above, were used, and the peroxide added directly to the tubes. When testing the viability of an organism, subcultures were made every thirty seconds until no further growth was obtained and subsequent in-

cubation showed the microorganism to have been dead.

Ozone was first generated in the laboratory by passing oxygen through a Siemen's tube and supplying the current and necessary voltage by means of batteries and induction coil. Later a single unit ozonizer (Gerard) was used and higher concentrations of ozone were made available. This ozonizer was built for ozonizing air and had a drier attached; however, by disconnecting the air pump and connecting with an oxygen tank, oxygen could be ozonized. Four factors influenced the concentration of ozone obtained with each apparatus: 1. The percentage of oxygen in the gas ozonized; 2, the rate at which the gas was passed through the ozonizer; 3, the electrical discharge and its potential; 4, the condition of the gas as regards temperature and moisture. With the Gerard apparatus the fixed factor was the third, the others were known and controllable; with the Siemen's tube the third factor was variable, influencing the output of ozone. The maximum concentration with the latter apparatus when working satisfactorily, was 0.001 per cent. by weight of ozone at 22° C. With the single unit ozonizer, the proportion of the ozone by weight could be varied between 0.005 and 0.012 per cent. when air was used, and between 0.015 and 0.0185 per cent. when oxygen was the gas ozonized. While using air the maximum amount of ozone that could be generated per minute at 22° C., was 15.6 milligrammes; while using ninety-five per cent. dried oxygen the maximum amount that could be generated per minute at 22° C. was twenty-four milligrammes. To secure dilutions between the 0.001 per cent. (first apparatus) and 0.005 per cent. (second apparatus) it would have been necessary to use large quantities of oxygen and to pass the gas quite rapidly over the electrodes; but, aside from the waste of oxygen entailed, the rate of flow of the gas was too rapid for convenient use in connection with the experiments. The test employed for estimating

the amount of ozone in a given specimen was the one ordinarily used, passing a known volume of gas through a neutral solution of potassium iodide, acidulating with sulphuric acid, titrating the iodine with sodium thiosulphate, using starch solution as an indicator. For the work with ozone all tubing had to be of glass or of special aluminum make, and all corks and joints rendered free from leakage by means of electrician's tape or paraffin. To approximate the intimate manner in which oxygen could be passed through a suspension of bacteria in a test tube (i. e. by passing the oxygen through a sterile hard rubber tube having many fine perforations in its sides near the end), wash bottles with specially made double glass stoppers were used; these permitted the ozone to enter at the lowest portion of the suspension from a glass bulb in which there were several fine perforations, with free exit for gas from the bottle through the outer cork.

OXYGEN.

Each one of the four microorganisms was grown on solid media in litre and half litre flasks, and the atmosphere in each flask replaced by one of ninety-five per cent. oxygen. The flasks, then rendered free from leakage, were kept at 37° C. Control flasks containing air were likewise rendered free from any external gas interchange and kept under the same conditions. Growth in the flasks containing oxygen seemed in each instance as luxuriant as in the control flasks. Different flasks were inoculated with the four different microorganisms and before visible growth had taken place their atmospheres were replaced by oxygen. These showed variable times of appearance and rates of growth for the first forty-eight hours in the aurococcus flasks, but good growths in all other flasks, the growth frequently being macroscopically visible in one of these flasks before that in the corresponding control. It may be observed here, that, using fresh media containing considerable moisture, the tubercle

bacillus seemed to grow as readily in the presence of oxygen as in the presence of air in the controls; but, given dry media and dried oxygen, growth could be inhibited while these conditions existed.

Fluid cultures of any of these bacteria, before incubation, were subjected to a continuous stream of oxygen for twelve, twenty-four, and forty-eight hours and showed at the end of these periods luxuriant growths in all tubes except those containing the tubercle bacillus. Here, there was an absence of any visible growth when the oxygen had been passed continuously through the suspension in the form of numerous fine bubbles. Incubation of the tubes after discontinuing the oxygen, however, showed the bacteria to have been simply inhibited in their growth and not killed. The aurococcus cultures appeared bleached but regained their normal color upon discontinuing the oxygen and transplanting into fresh media. Aside from this bleaching of the aurococcus and the absence of growth under the conditions mentioned in the tubes containing the tubercle bacillus, there was no effect observed upon the growth of any of the other bacteria. The streptococcus and the colon cultures not only remained viable, but grew luxuriantly while being exposed in fluid media to a continuous current of oxygen.

The results obtained by oxygenating freshly transplanted fluid cultures of these bacteria led to the oxygenating of sterile water suspensions. Fractions of a culture were used—finally one seventh of a culture. The colon bacillus and the streptococcus could be as easily subcultured as their controls at the end of forty-eight hours' exposure to a continuous current of oxygen; the aurococcus invariably showed fewer colonies developing on subculture from the oxygenated suspensions than from the controls. Cultures from the tubercle bacillus showed latent growth as compared with cultures from its control suspensions. The aurococcus was subjected for another twenty-four hours to the stream of oxygen, at the end of which time, seventy-

two hours, no growth was obtained on testing for viability, whereas light growths of aurococci could still be obtained from the controls.

The action of ninety-five per cent. oxygen on any of these microorganisms in the presence of culture media did not demonstrate it to be bactericidal in any instance, and only in the case of one organism —that of the tubercle bacillus—did it inhibit growth; i. e., when the bacteria were freshly transplanted into fluid media and subjected to the action of the oxygen in a continuous current passed through the culture. Discontinuation of the oxygen and further incubation renewed the growth of the bacilli.

In the absence of organic matter, on the other hand, oxygen used as a continuous stream of fine bubbles passed through a freshly suspended fraction of a culture in sterile water, proved inhibitory to the subsequent growth of the aurococcus and the tubercle bacillus. In the latter case the tubercle bacilli in suspension had to be first thoroughly emulsified, as the presence of bacilli in clumps in the suspensions seemed to protect those within the clump. No influence on the viability of *Streptococcus brevis* or of *Bacillus coli* was observed upon similar subjection to oxygen for forty-eight hours. With prolonged use of the oxygen, i. e., for at least seventy-two hours or until such time as the gas had been brought into intimate contact with the bacteria in suspension, no growth or subculture could be obtained with the aurococcus. Control suspensions could be subcultured but had not been subjected to continuous agitation by means of a current of sterile air.

HYDROGEN DIOXIDE.

The results with this oxidizing agent varied with the two different makes that were used, though both were labeled, "U. S. P. three per cent." For convenience they will be designated as (A) and (B). Employing suspensions of the bacteria in sterile water, the amount of hydrogen dioxide (A) which

would kill one culture of the aurococcus in five minutes, when added to it, was one third of the amount required when using the hydrogen dioxide (B). The amount of hydrogen dioxide (A) which would kill one culture of either *Streptococcus brevis* or *Bacillus coli* in five minutes was just one half the amount required of the hydrogen dioxide (B). Any fluid culture of any one of the different bacteria could be killed within the same length of time—five minutes—that was required to kill suspensions in sterile water, but never with as small amounts of the hydrogen dioxide as proved sufficient in the suspensions. The same was true with cultures on solid media, greater amounts were required to kill a given culture than when in aqueous suspension; and proportionately greater amounts of the hydrogen dioxide (B) were necessary than of hydrogen dioxide (A). Apparently part of the oxidizing agent was reduced by the media.

OZONE.

Passing from hydrogen dioxide to ozone—another oxidizing agent, from which nascent oxygen is derived at the moment of its liberation in the atomic state—results with its use were dependent entirely upon the intimacy and rapidity with which it could be brought into contact with the bodies of the bacteria suspended in fluid media. With the first apparatus used for generating ozone, the Siemen's tube, though the proportion of ozone by weight in the ozonized oxygen was never greater than 0.001 per cent., suspended cultures of the four different microorganisms could be killed if exposed to its action for a sufficient length of time. Or, in other words, the passage of as many litres of this strength of ozone through a culture (suspended in sterile water) as would represent at the maximum the use of 37.5 milligrammes of ozone, was sufficient to kill any one culture of bacteria. Twenty milligrammes was the smallest amount, with this concentration, which was ever found to kill a cul-

ture. When the use of greater concentrations of ozone was possible, as with the single unit ozonizer, any one culture of the aurococcus, *Streptococcus brevis,* or *Bacillus coli* could be killed by two minutes' exposure to ozonized air containing 0.012 per cent. by weight of ozone; or could be killed in one minute by exposure to ozonized oxygen containing from 0.015 to 0.0185 per cent. of ozone with an unused surplus of ozone invariably remaining, as shown by tests for ozone in the escaping gas. Ozone gas, whether used at 22° C. or with its temperature raised to 33° C., seemed to give equally good results as regards the rapidity with which the bacteria could be killed, once the ozone was brought into intimate contact with them in fluid suspension; less ozone being required in the absence of organic matter.

Cultures on solid culture media could be killed by using greater amounts of ozonized air or ozonized oxygen. This was always, however, a difficult procedure, as more ozone escaped from the vessel than was reduced by the bacteria and the media. Invariably subcultures could be obtained from the centre of colonies and from that portion of growths next to the surface of the media, long after those from the periphery and surface of the colony had ceased to grow on transplantation. Only by the use of ozone in high concentration and under pressure could bacteria within a solid medium be reached and killed by the gas.

Ozone proved to be an active bactericide *in vitro* for all the microorganisms used, provided certain conditions were fulfilled. The bacteria had to be in fluid culture or in suspension in a fluid medium such as water, and the gas had to be passed through the suspension or medium rapidly, in the form of numerous fine bubbles. It was necessary to break up the heavy film growth of the tubercle bacillus to secure the rapid oxidation of the latter. Ozone had no selective action as regards these bacteria in the presence of organic matter, such as that con-

tained in the culture media used. To kill one culture of any of the bacteria in sterile water suspension, the maximum amount of ozone used was 37.5 milligrammes (0.001 per cent. strength), the minimum, twelve milligrammes (0.005 to 0.012 per cent. concentration). To kill one culture in a fluid medium of any of the bacteria mentioned, the maximum amount of ozone used was 70.5 milligrammes, the minimum twenty milligrammes.

CONCLUSIONS.

1. Oxygen in its molecular form O_2, ninety-five per cent. pure, does not kill or inhibit the growth in the moist state of *Streptococcus brevis* or of *Bacillus coli.*

2. Oxygen may inhibit the growth of the tubercle bacillus when brought into intimate contact with each microorganism in the moist state, but does not kill it.

3. In the absence of culture media (organic matter), oxygen inhibits the growth of the aurococcus, and may kill it, after prolonged and intimate contact with each microorganism in the moist state.

4. Hydrogen dioxide (U. S. P. three per cent.) is an active bactericide for the four microorganisms tested. The action is dependent upon the amount of the hydrogen dioxide used, the age of the preparation, its strength, and, also upon the presence or absence of readily oxidizable organic matter.

5. Ozone (ozonized air or ozonized oxygen) under certain given conditions is an active bactericide for the four microorganisms used. Its action is dependent upon its concentration and the intimacy and rapidity with which it is brought into direct contact with the bodies of all the bacteria in suspension in water or fluid media.

6. Ozone does not dissolve in sterile water, or in saline solution in more than faint traces; therefore its bactericidal action is dependent upon its passage through these solutions or through any fluid me-

dium as a continuous stream of numerous fine bubbles of gas.

7. Ozone has no selective action for any of the four bacteria in the presence of organic matter.

REFERENCES:

1. BAINBRIDGE, W. S.: *New York State Journal of Medicine*, vi, p. 281, 1908. 2. MEEKER, H. D.: NEW YORK MEDICAL JOURNAL, lxxxix, p. 741, 1909. 3. BAINBRIDGE, W. S.: *Annals of Surgery*, 1909, p. 305.