Effect of Hyperbaric Oxygen on Clostridia *in vitro* and *in vivo*.* (31743)

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Recent reports(1-6) have described the treatment of humans with clostridial gas gangrene and tetanus in hyperbaric oxygen chambers. Although most of these reports attribute beneficial results to use of hyperbaric oxygen none were controlled studies. The evidence for efficacy of hyperbaric oxygen in therapy of clostridial infection is sparse. Furthermore, hyperbaric oxygen has serious toxicities in man(7). In view of the widespread enthusiasm concerning hyperbaric oxygen therapy for clostridial infections, it is essential to obtain adequately controlled laboratory observations on the effect of hyperbaric oxygen in experimental infection.

The purpose of this research was to study by quantitative techniques, the effect of oxygen under pressure on clostridia *in vitro* and to investigate the influence of oxygen therapy on *C. perfringens* infections in animals.

Materials and methods. Clostridium perfringens (American Type Culture Collection 8009) and *Clostridium tetani* (American Type Culture Collection 9441) were studied. All cultures were incubated anaerobically in a hydrogen atmosphere. Stock cultures were maintained by lyophilizing aliquots of a 24 hour trypticase soy broth culture. For each experiment an aliquot of stock culture was subcultured to trypticase soy broth and incubated at 37°C for 24 hours (24 hour culture). Four hour cultures were prepared by diluting a 24 hour culture 1:10,000 in trypticase soy broth and incubating for 4 hours at 37°C. Numbers of bacteria were determined by serially diluting in trypticase soy broth and making pour plates with trypticase soy agar. All plates were read after incubation for 48 hours at 37°C.

In vitro *experiments. In vitro* experiments were performed in Torbal-B.T.L. Anaerobic Jars (Torsion Balance Co.) which were fitted with pressure gauges and could be pressurized to 2 atmospheres absolute or 30 lb per square inch absolute (30 p.s.i.a). Air was eliminated from the jars by alternately evacuating with negative pressure and filling with the appropriate gas under pressure. Suspensions of bacteria in trypticase soy broth in covered petri dishes were incubated in air or oxygen at 15 p.s.i.a., oxygen at 30 p.s.i.a., or nitrogen at 15 or 30 p.s.i.a. These provided respectively environments with oxygen at about 150 mm Hg, 760 mm Hg and 1520 mm Hg and anaerobic environments at atmospheric pressure and twice atmospheric pressure. The broth was only 1.5-2.5 mm deep to allow diffusion of gas. Experiments were performed at 37°C unless otherwise specified. Periodically aliquots of culture medium were removed and the numbers of viable microorganisms determined.

In vivo *studies.* Male Swiss mice of the CF1 strain (Carworth Farms) weighing 20 to 24 g were used in all experiments and were allowed food and water *ad lib* when not in the hyperbaric chamber. To produce infection, *C. perfringens* from a 24 hour culture were washed and suspended in saline solution and injected in volumes of 0.2 ml intramuscularly in the ham string muscles of the right hind leg. Following infection, half of the mice in each group were exposed to 100% oxygen at 45 p.s.i.a. for 30 minutes every 8-16 hours for 3-6 treatments in a Vickers Tank Type Hyperbaric Oxygen Chamber; the other half of each group was not exposed to oxygen and served as controls. Mice were removed from the chamber between exposures. The first exposure to oxygen was always started within 90 minutes of initiation of infection. With each exposure 10 minutes were allowed for

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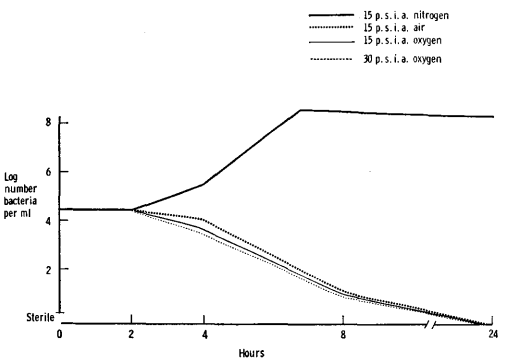


FIG. 1. Effect of oxygen on *Clostridium perfringens*. Number of *C. perfringens* per ml of trypticase soy broth following incubation at 37°C of 24 hr cultures in nitrogen, air, or oxygen at 15 lb per square inch absolute (15 p.s.i.a.) or oxygen at 30 p.s.i.a.

compression and 10 minutes for decompression; therefore, total time in the chamber was 50 minutes for each exposure. At operating pressure the exchange rate of oxygen in the chamber was about 114 liters per minute (12 standard cubic feet per minute).

Results. In vitro *experiments.* Exposure of *C. perfringens* in trypticase soy broth to air or oxygen at 15 p.s.i.a. or to oxygen at 30 p.s.i.a. all resulted in a marked and equivalent bactericidal effect. Fig. 1 demonstrates the results of a typical experiment. In this and additional experiments there was no significant difference between the rate of sterilization in air at 15 p.s.i.a., oxygen at 15 p.s.i.a., or oxygen at 30 p.s.i.a. In all experiments *C. perfringens* was incubated in nitrogen as a control. The microorganisms always multiplied in nitrogen and multiplication was equal in nitrogen at 15 p.s.i.a. or 30 p.s.i.a.

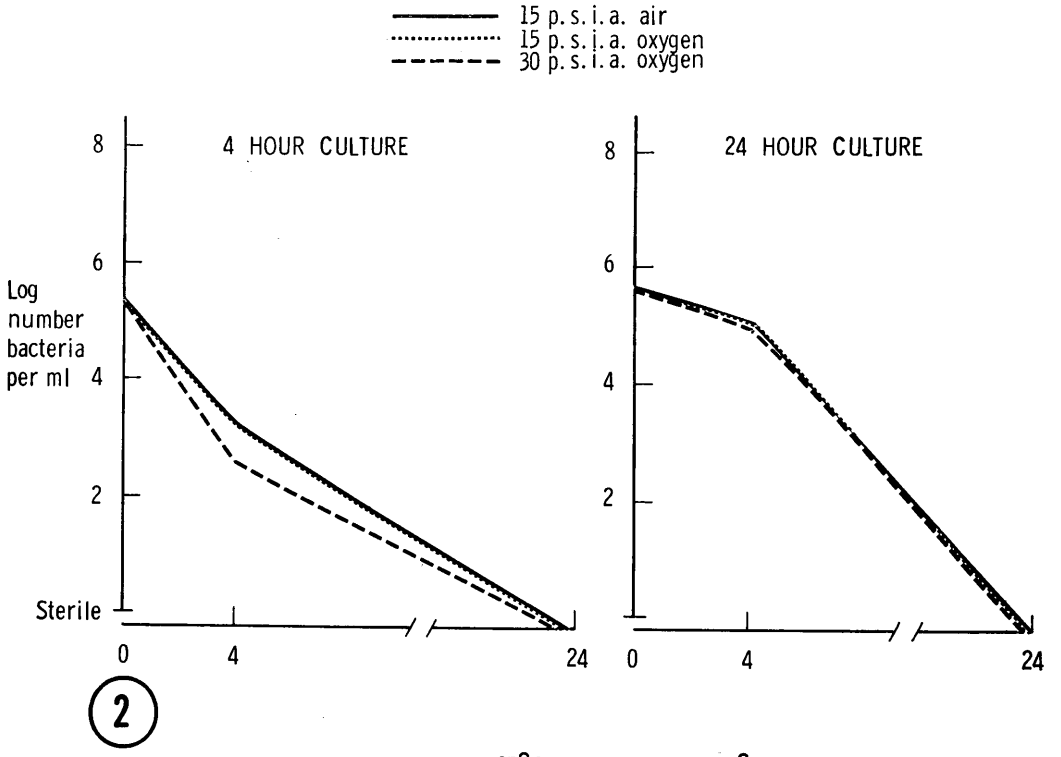
The age of the culture, temperature of incubation, and presence of blood or muscle all influenced the bactericidal effect of oxygen. Exposure to air or oxygen resulted in a more rapid bactericidal effect in 4 hour cultures than in 24 hour cultures (Fig. 2); the rate of killing of *C. perfringens* by oxygen was less at 25°C than at 37°C (Fig. 3); the presence of 10% defibrinated sheep blood or heparinized human, rabbit or mouse blood in trypticase soy broth inhibited the bactericidal activity of oxygen (Fig. 4); and normal ham string muscle from rabbits or mice homogenized in a teflon tissue grinder (Scientific Glass Apparatus Co.) and suspended in trypticase soy broth inhibited the bactericidal activity of oxygen (Fig. 5).

Exposure to oxygen of *C. perfringens* streaked on the surfaces of agar plates also resulted in inhibition of growth. In these experiments 4 or 24 hour trypticase soy broth cultures of *C. perfringens* were streaked on the surfaces of trypticase soy agar plates (with and without 5% sheep blood). Plates were: (1) incubated anaerobically in hydrogen; (2) exposed to oxygen at 30 p.s.i.a. for 5 hours at 37°C and then incubated anaerobically in hydrogen; or (3) exposed to oxygen at 30 p.s.i.a. for 24 hours at 37°C and then incubated anaerobically in hydrogen. Exposure of cultures on plain agar to oxygen for 5 hours inhibited subsequent growth of 4 hour cultures more than growth of 24 hour cultures. Exposure of cultures on plain agar to oxygen for 24 hours completely inhibited subsequent growth of both 4 and 24 hour cultures. Presence of 5% sheep blood in the agar markedly inhibited the antibacterial action of oxygen.

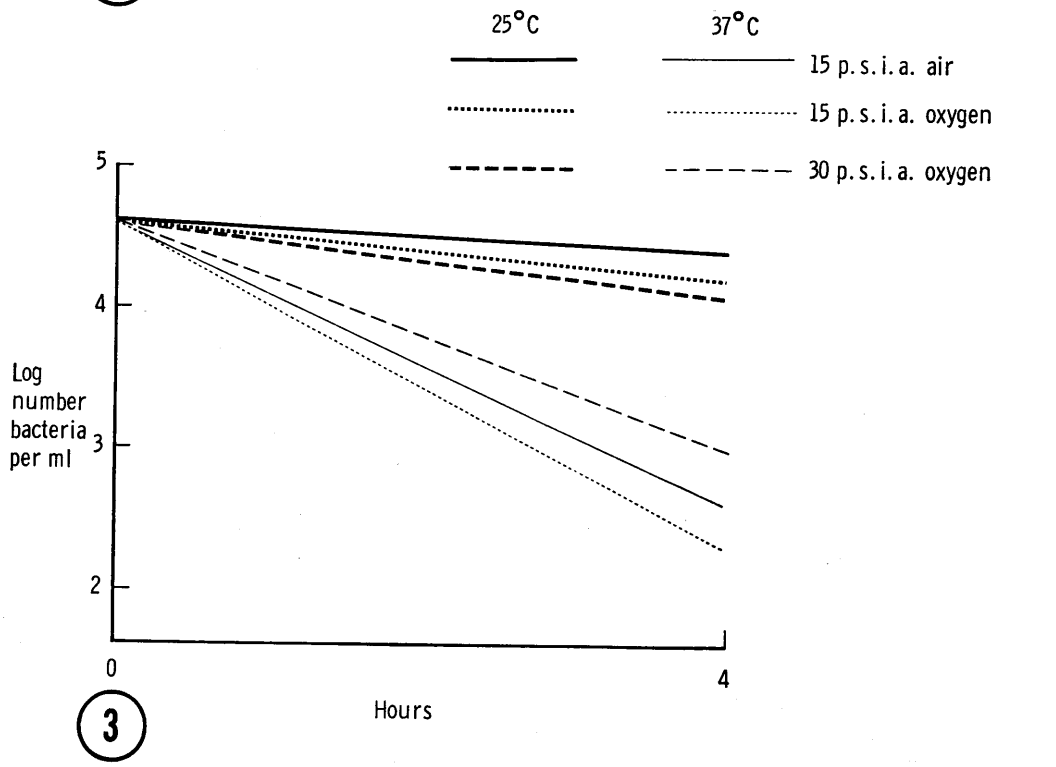
Oxygen was also bactericidal for *C. tetani.* Exposure of trypticase soy broth cultures to air at 15 p.s.i.a. or oxygen at 15 or 30 p.s.i.a. resulted in a marked bactericidal effect similar to that observed with *C. perfringens.* Furthermore, 4 hour cultures were more susceptible to the bactericidal action of oxyygen than were 24 hour cultures.

In vivo *experiments.* Treatment with hyperbaric oxygen protected mice against mortality from infection with *C. perfringens.* Table I demonstrates typical experiments in which the inoculum used was lethal for about 50% of mice not exposed to oxygen but killed only 15 to 25% of mice treated with oxygen ($P < .01$). There was no mortality in groups of uninfected mice exposed to oxygen in the chamber simultaneously with the mice infected with *C. perfringens.* In all experiments with *C. perfringens* death usually occurred within 72 hours after infection and mortality was calculated 14 days following infection.

To simulate infection in traumatic wounds containing foreign material, the ham string muscles of the right hind leg were crushed with padded hemostats for one minute and


15 p.s.i.a. air
15 p.s.i.a. oxygen
30 p.s.i.a. oxygen
4 HOUR CULTURE
24 HOUR CULTURE
Log number bacteria per ml
8
6
4
2
Sterile
0
4
24

2


25°C
37°C
15 p.s.i.a. air
15 p.s.i.a. oxygen
30 p.s.i.a. oxygen
Log number bacteria per ml
5
4
3
2
0
4
Hours

3

FIG. 2. Effect of age of the culture on bactericidal activity of oxygen against *Clostridium perfringens*. Number of *C. perfringens* per ml of trypticase soy broth following incubation at 37°C of 4 or 24 hr cultures in air, or oxygen at 15 p.s.i.a. or oxygen at 30 p.s.i.a.

FIG. 3. Effect of temperature on bactericidal activity of oxygen against *Clostridium perfringens*. Number of *C. perfringens* per ml of trypticase soy broth following incubation at 25°C or 37°C of 4 hr cultures in air or oxygen at 15 p.s.i.a. or oxygen at 30 p.s.i.a.

C. perfringens mixed with infusorial earth (Fisher Scientific Co., Pittsburg, Pa.) in 0.2 ml saline solution was injected into the crushed area. As shown in Table II, mice exposed to oxygen were significantly protected from mortality ($P<.01$) when compared to control groups. There was no mortality in mice treated by crushing the muscles and/or injection of infusorial earth with or without subsequent exposure to oxygen.

Certain variations in the experimental model eliminated the protective effect of oxy-

TABLE I. Results Following Injection of *C. perfringens* in Normal Muscle.

No. of exposures to oxygen	Inoculum	Exposed to oxygen: Mice surviving*/Total inj	(%)	Not exposed to oxygen: Mice surviving*/Total inj	(%)
3	1×10^8	17/20	(85)	10/20	(50)
5	1×10^8	15/20	(75)	11/20	(55)
	Totals	32/40†	(80)	21/40†	(53)

* Calculated 14 days after infection. † $P < .01$ by chi square analysis.

TABLE II. Results Following Injection of *C. perfringens* and Infusorial Earth in Crushed Muscle.

No. of exposures to oxygen	Inoculum	Exposed to oxygen: Mice surviving*/Total inj	(%)	Not exposed to oxygen: Mice surviving*/Total inj	(%)
3	4×10^6	10/11	(90)	4/11	(36)
4	1.5×10^7	21/22	(95)	14/22	(64)
5	1×10^7	11/14	(79)	8/15	(53)
6	7×10^6	11/11	(100)	8/11	(73)
6	2.8×10^7	9/10	(90)	7/10	(70)
	Totals	62/68†	(91)	41/69†	(59)

* Calculated 14 days after infection. † $P < .01$ by chi square analysis.

TABLE III. Results Following Injection of *C. perfringens* With or Without Calcium Chloride or Infusorial Earth in Normal or Crushed Muscle.

Procedure: Muscle crushed	Calcium chloride	Infusorial earth	Inoculum	No. of exposures to oxygen	Exposed to oxygen: Mice surviving*/Total injected	(%)	Not exposed to oxygen: Mice surviving*/Total injected	(%)
No	No	Yes	6×10^7	4	21/25	(84)	20/24	(83)
No	Yes	No	3×10^7–9×10^7	3–5	24/55	(44)	22/55	(40)
No	Yes	No	2×10^5–8×10^5	3–5	85/135	(63)	87/135	(64)
Yes	Yes	No	3×10^7–9×10^7	3–5	33/50	(66)	30/50	(60)
Yes	Yes	No	8×10^5	4	26/31	(84)	29/31	(94)
Yes	No	No	8×10^7	4	7/11	(64)	6/11	(55)

* Calculated 14 days after infection.

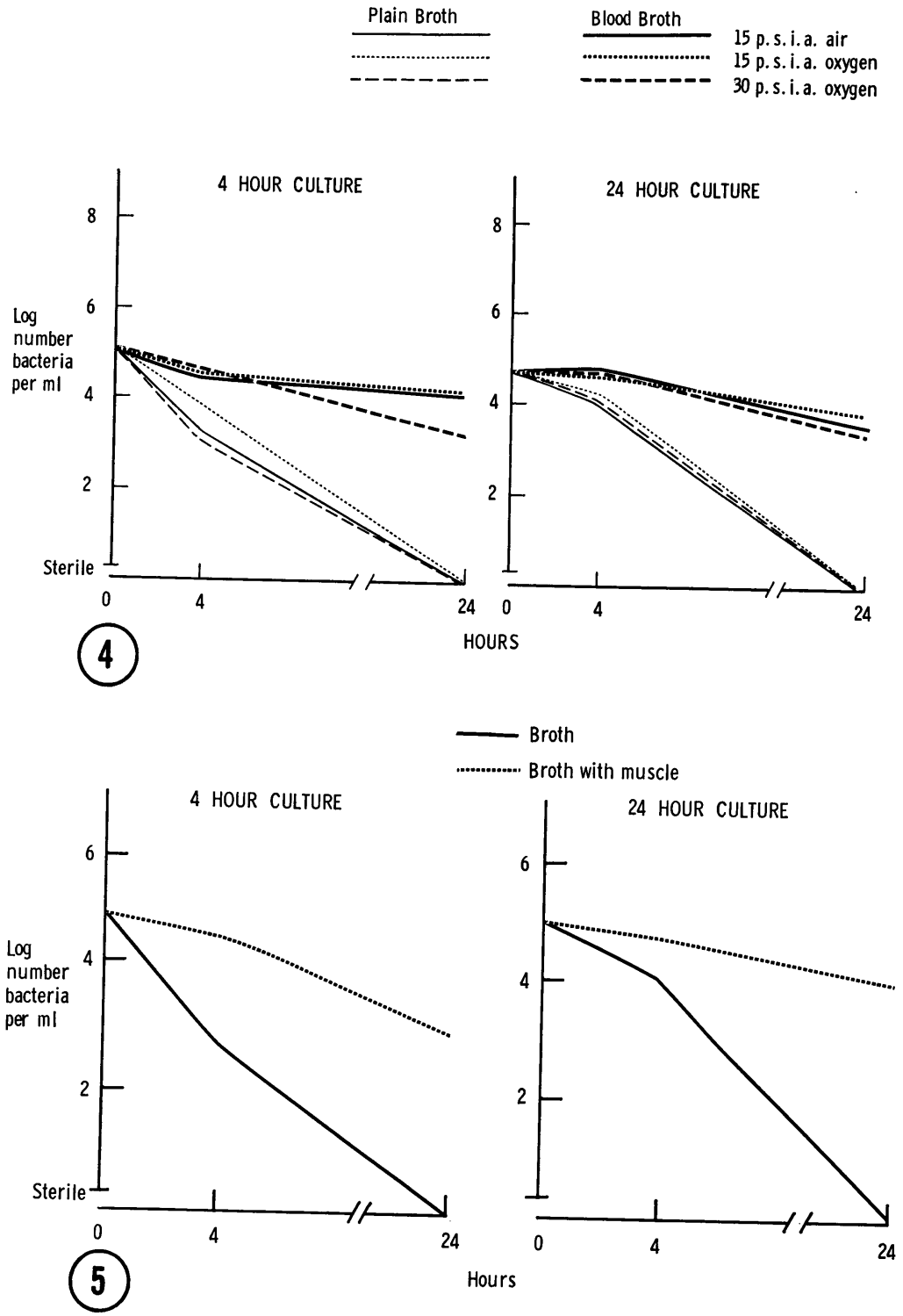


FIG. 4. Effect of blood on bactericidal activity of oxygen against *Clostridium perfringens*. Number of *C. perfringens* per ml of trypticase soy broth or 10% sheep blood in trypticase soy

broth following incubation at 37°C of 4 or 24 hr cultures in air or oxygen at 15 p.s.i.a. or oxygen at 30 p.s.i.a.

FIG. 5. Effect of muscle on bactericidal activity of oxygen against *Clostridium perfringens*. Number of *C. perfringens* per ml of trypticase soy broth or 10% homogenized rabbit muscle in trypticase soy broth following incubation at 37°C of 4 or 24 hr cultures in oxygen at 30 p.s.i.a.

gen. Exposures to oxygen at 45 p.s.i.a. identical to those in Table I and II did not significantly protect mice from death following injection into normal ham string muscles of *C. perfringens* mixed with infusorial earth or *C. perfringens* suspended in 5% calcium chloride solution (an agent which enhances pathogenicity of *C. perfringens*). Similarly oxygen therapy did not significantly protect mice from mortality following injection into crushed ham string muscles of *C. perfringens* suspended in 5% calcium chloride solution or *C. perfringens* alone. Table III demonstrates the results of some of these experiments. The various procedures listed in Table III with or without subsequent exposure to oxygen did not result in any deaths in mice not challenged with clostridia.

Death was associated with multiplication of *C. perfringens* in the muscle. Mice were injected intramuscularly with lethal inocula of *C. perfringens* with or without 5% calcium chloride. Groups of 12 mice were sacrificed immediately after the injection or 6 hours after injection. The injected legs were resected, skinned and each homogenized in 1 ml trypticase soy broth in a teflon tissue grinder. Determination of the number of bacteria present revealed at least a 10-fold increase in the titer of clostridia during the 6 hour interval.

Discussion. In an abstract, Nuckolls and Osterhout(8) reported that log phase cultures of *C. perfringens* were more rapidly inactivated by oxygen than stationary phase cultures and that the lethal effect of oxygen on *C. perfringens* in solid media was reduced by the presence of blood. The present studies confirm and extend these observations. Blood in both liquid and solid media, muscle in liquid media, and a decreased temperature of incubation (25°C) markedly decreased the bactericidal action of oxygen for *C. perfringens*.

Although *C. perfringens* is highly susceptible to the bactericidal action of oxygen *in vitro*, strains of *C. perfringens* sometimes remain viable in wounds in humans despite therapy with hyperbaric oxygen(9). This finding may be explained in part by factors present *in vivo* that function to decrease the bactericidal activity of oxygen for clostridia: (1) blood and muscle are present in wounds; (2) the temperature of an avascular wound might be below 37°C; (3) the amount of oxygen actually delivered to an infected site depends upon integrity of the blood supply and may be low despite hyperbaric oxygenation.

There has been varied success in protecting animals from infection caused by *C. perfringens* with hyperbaric oxygen therapy. The greater protection of oxygen therapy found by Kelley and Pace(10), Klopper(11) and in the present study as compared with the lack of protection found by others (2,12,13) may be explained in part by differences in bacterial or animal strains or by differences in experimental technique. The present study demonstrates that small changes in the design of the experiment can eliminate the protective effect of oxygen on *C. perfringens* infection in mice; (*i.e.*, no protective effect was demonstrated following injection of calcium chloride instead of infusorial earth in crushed muscle or following injection of calcium chloride or infusorial earth in normal muscle). The reason for the absence of a protective effect of oxygen with these changes in experimental design is unknown.

It is likely that the experimental model used (*e.g.*, crushing muscle or injecting calcium chloride) greatly modifies the local tissue oxygen tension by altering blood flow, and perhaps altering vascular permeability, oxygen diffusion and tissue metabolism. Therefore, even with hyperbaric oxygen treatment of the mouse, the oxygen tension at the site of clostridial infection could vary greatly with the experimental model employed.

Summary. Exposure of broth cultures of *C. perfringens* to air or oxygen at 15 pounds

per square inch absolute (15 p.s.i.a.) or to oxygen at 30 p.s.i.a. resulted in a marked and equivalent bactericidal effect. Four hour cultures were more susceptible to the bactericidal action of oxygen than 24 hour cultures. The rate of inactivation of *C. perfringens* by oxygen was decreased by the presence of blood or muscle and was less at 25° than at 37°C. Exposure to oxygen of *C. perfringens* on the surfaces of agar plates resulted in inhibition of growth on subsequent incubation in an anaerobic environment. Four hour cultures were more susceptible to the inhibitory activity of oxygen than 24 hour cultures and presence of blood in the agar inhibited the antibacterial action of oxygen. Exposure of broth cultures of *C. tetani* to air or oxygen at 15 p.s.i.a, or to oxygen at 30 p.s.i.a., resulted in a bactericidal effect similar to that observed with *C. perfringens*.

Mice injected intramuscularly in normal muscle with *C. perfringens* could be protected against mortality with treatment with oxygen at 45 p.s.i.a. Oxygen also protected against death in experiments in which infusorial earth was injected with *C. perfringens* into crushed muscle. Variations in the experimental model such as injecting calcium chloride instead of infusorial earth with *C. perfringens* into normal or crushed muscle eliminated the protective effect of oxygen.

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Antibodies to Mycoplasma in Sera of Leukemic Patients.* (31744)

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The experiments reported were done to determine whether virus neutralization technics were useful for testing patients' sera for antibodies that neutralized the cytopathic effect (CPE) and growth of mycoplasma (PPLO) isolated from bone marrow specimens obtained from leukemic and non-leukemic patients. Results also are presented from pilot studies carried out to test whether a correlative pattern existed between leukemia and the occurrence of antibodies to such mycoplasma. The findings are of interest because evidence is presented that a complement dependent normal antibody is present in serum that neutralizes PPLO CPE and growth. The problem of interpreting results is discussed when serum specimens are obtained from patients with a disease that causes marked pathologic changes in antibody forming tissues.

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Materials and methods. Cell lines. The