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Hydrogen Peroxide and Superoxide Radical Formation in Anaerobic Broth Media Exposed to Atmospheric Oxygen

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Fourteen different broth media were autoclaved under anaerobic conditions and then exposed to atmospheric oxygen. The hydrogen peroxide and superoxide radical formation as well as the bactericidal effect of the media were studied. The rate of killing of *Peptostreptococcus anaerobius* VPI 4330-1 was high in media that rapidly autoxidized and accumulated hydrogen peroxide. In actinomyces broth (BBL), 50% of the cells were killed within 2 min, and in Brewer thioglycolate medium (Difco), 50% were killed within 11 min, whereas more than 50% of the cells survived for more than 2 h in Clausen medium (Oxoid), fluid thioglycolate medium (BBL), and thioglycolate medium without dextrose or indicator (Difco). Only media that contained phosphate and glucose had a tendency to accumulate hydrogen peroxide. A solution of phosphate and glucose autoxidized when it had been heated to 120°C for at least 5 min and when the pH of the solution was higher than 6.5. Transitional metal ions catalyzed the autoxidation, but they were not necessary for the reaction to occur. Of the other substances heated in phosphate buffer, only α-hydroxycarbonyl compounds autoxidized with accumulation of hydrogen peroxide. Superoxide dismutase decreased the autoxidation rate of most of the broth media. This indicated that superoxide radicals were generated in these media.

Reduction of molecular oxygen to water usually proceeds by a series of single electron transfers, which generate the reactive intermediates hydrogen peroxide, superoxide radicals, and hydroxyl radicals (12). The bactericidal effect of hydrogen peroxide for anaerobes has been recognized for a long time (4, 25), and the excellent protecting effect of catalase has been demonstrated (5, 6, 13, 17, 18, 28). More recently, the importance of superoxide and hydroxyl radicals as bactericidal agents has been recognized (15, 23).

The formation of hydrogen peroxide in the autoxidation of bacteriological culture media has been known for some time (1, 6, 13, 28). A generation of superoxide or hydroxyl radicals in the media has not been demonstrated. The aim of the present investigation was to study the formation of hydrogen peroxide and superoxide radicals in the autoxidation of various anaerobic broth media and to identify components of the media responsible for the formation of these intermediates of oxygen reduction.

MATERIALS AND METHODS

Microorganism. *Peptostreptococcus anaerobius* VPI 4330-1 (ATCC 27337) was used as the test organism. It was kept on blood agar plates at 4°C under strictly anaerobic conditions in an anaerobic box with an atmosphere of 10% H_2 and 5% CO_2 in nitrogen (36).

Chemicals. The commercial sources of the broth media are given in Table 1. Catalase (purified powder from beef liver, C10, Sigma Chemical Co., St. Louis, Mo.) was purified from superoxide dismutase (SOD) activity by Sephadex G 200 (Pharmacia, Uppsala, Sweden) gel filtration in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.1 M KCl. One milligram of protein of the purified catalase preparation decomposed 25 mmol of hydrogen peroxide per min at 25°C and pH 7.0. SOD was from Sigma Chemical Co. The SOD preparation contained 2,700 U/mg as defined by McCord and Fridovich (22). No catalase activity could be detected in the SOD preparation when tested according to Beers and Sizer (3). Horse blood (GIBCO, Bio-Cult Ltd., Paisley, Scotland) was hemolyzed by freeze-thawing. This blood was used in the blood agar medium (19).

Preparation of media. Peptone-yeast extract (PY)-glucose broth and dilution blanks (0.2% gelatin, salts, resazurin) were prepared as described by Holdeman and Moore (19). Other broth media and other solutions were prepared in the anaerobic box by dissolving the dehydrated media ingredients in oxygen-free water. The water was made oxygen free by bringing hot, just autoclaved, water into the box, where it was kept for at least 1 day before use. The broth media were brought out from the box in tightly stoppered test tubes, autoclaved at 120°C, and cooled to 25°C in a water bath.

Bactericidal effect of broth media exposed to atmospheric oxygen. *P. anaerobius* VPI 4330-1 was

TABLE 1. *Parameters of anaerobic broth media exposed to atmospheric oxygen*[a]

Media[b]	Time to 50% survival of *P. anaerobius* (min)	Autoxidation rate (nmol of O_2 consumed/ml per min)	Formation of superoxide radicals[c] (%)	Accumulated H_2O_2[d] (μM)	Phosphate concn (mM)	Glucose concn[e] (mM)
Actinomyces broth (BBL)	2	152	39	73	112	28
Brewer thioglycolate medium (Difco)	11	101	23	0	22	28
Brain heart infusion (BBL)	28	21	24	18	17	11
Trypticase soy broth (BBL)	48	21	21	11	15	14
Indole-nitrite medium (BBL)	119	7	48	4	14	6
Schaedler broth (BBL)	19	40	24	26	8.2	32
Mycoplasma broth base (BBL)	108	3	-[f]	4	2.7	0
Clausen medium (Oxoid)	>120	28	20	0	2.3	33
Fluid thioglycolate medium (BBL)	>120	12	32	0	2.2	28
Thioglycolate medium (Brewer) (Oxoid)	27	8	26	3	1.9	28
Thioglycolate medium without dextrose or indicator (Difco)	>120	15	19	0	1.0	0
Phenol red broth base (Difco)	>120	0	-	0	0.6	0
Dextrose broth (Difco)	75	5	25	4	0.4	28
PPLO broth without crystal violet (Difco)	>120	0	-	0	0.4	0

[a] The following media did not autoxidize: Lab Lemco powder (Oxoid), vitamin-free Casamino Acids (Difco), beef heart infusion (Difco), peptone (Difco), Trypticase (BBL), proteose peptone (Oxoid), neutralized bacteriological peptone (Oxoid), tryptone (Difco), Phytone (BBL), tryptose (Difco), Casitone (Difco), soya peptone (Oxoid), Thiotone (BBL), and yeast extract (Difco). They were heated in 1% solutions for 30 min at 120°C.

[b] Media were heated for 30 min at 120°C.

[c] Percentage of inhibition of autoxidation rate in the presence of SOD.

[d] After 15 min of exposure to oxygen.

[e] According to manufacturer.

[f] -, Rate of autoxidation was too slow to be accurately determined.

grown at 37°C in PY-glucose broth in the anaerobic box. When the culture reached a turbidity of 0.5 at 600 nm, it was diluted in 10-fold steps in dilution blanks without added cysteine. A 20-μl sample of dilution 10^{-3} was inoculated at ambient temperature into a screw-capped test tube containing 2 ml of the broth medium to be tested. The density of cells in the broth was around 3,000 cells per ml. The test tube was taken from the anaerobic box, and the cell suspension was poured into a 50-ml round-bottomed flask submerged under continuous shaking into a thermostated (25°C) water bath. Samples (0.1 ml) were taken at regular time intervals from the flask and were spread over the surface of duplicate blood agar plates. Just before use, the plates were taken from the anaerobic box. The plates were returned to the box immediately after inoculation. The plates were incubated for 1 day at 37°C in the box, and the number of surviving cells after various times of oxygen exposure was determined.

Oxygen consumption. Oxygen consumption was studied in a biological oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio). The temperature of the system was kept at 25°C by a circulating water pump. The broth media to be tested were prepared in double strength and autoclaved for 30 min at 120°C. To 4 ml of water, saturated with atmospheric oxygen, in the chamber of the oxygen monitor, 4 ml of the broth medium was added under the protection of a flow of nitrogen gas, and the chamber was immediately closed by the oxygen electrode. The oxygen consumption was followed for 15 min or until the oxygen concentration in the mixture reached 15% saturation. The rate of oxygen consumption was expressed as nanomoles of oxygen consumed per minute per milliliter of double-strength broth during 15 min or until the oxygen concentration in the reaction mixture reached 15% saturation. Oxygen consumption of various compounds autoclaved in potassium phosphate buffer was studied in a similar way.

Hydrogen peroxide accumulated in various broth media. The broth medium (2 ml) was taken from the anaerobic box in a screw-capped test tube. The broth was poured into a 50-ml round-bottomed flask (25°C) under continuous shaking. After various time intervals of exposure to atmospheric oxygen, the broth was transferred into the chamber of the oxygen monitor. The chamber was immediately closed by the electrode, and the oxygen consumption was followed for 5 min. Then 5 μl of catalase (250 μg) was added, and the amount of oxygen evolved was determined. The concentration of hydrogen peroxide in the solution was determined by comparing this amount of oxygen evolved with the amount evolved after a subsequent addition of a standard amount of hydrogen peroxide. The concentration of hydrogen peroxide in the standard solution was determined by measuring E_{240} (ϵ_{mM}:43.2).

Hydrogen peroxide accumulated in the autoxidation of various compounds in potassium phosphate buffer was determined after the oxygen consumption had been followed for 15 min or the oxygen concentration of the reaction mixture had reached 15% saturation.

Formation of superoxide radicals in broth media. The oxygen consumption by the broth medium was determined in the presence and in the absence of SOD (2.5 μg/ml). A slower rate of oxygen consumption in the presence of SOD indicated formation of superoxide radicals in the autoxidation of the broth medium. SOD heated to 100°C for 10 min was used as control.

The broth media rapidly reduced cytochrome *c* as well as nitroblue tetrazolium in the absence of oxygen. It was not possible to measure the formation of superoxide radicals by the reduction of these compounds in the presence of oxygen.

Other methods. Phosphate was determined as described by Fiske and SubbaRow (11) and protein as described by Lowry et al. (21).

RESULTS

There were big differences in the bactericidal effects of the various broth media exposed to oxygen. In actinomyces broth (BBL), 50% of the *P. anaerobius* VPI 4330-1 cells were killed within 2 min, whereas more than 50% of the cells survived for more than 2 h in Clausen medium (Oxoid), fluid thioglycolate medium (BBL), thioglycolate medium without dextrose or indicator (Difco), phenol red broth base (Difco), and PPLO broth without crystal violet (Difco) (Table 1). The killing rate of the cells was often high in the media that rapidly autoxidized. The tendency of the media to autoxidize and accumulate hydrogen peroxide was usually related to the concentration of phosphate in the media (Table 1). SOD significantly reduced the rate of oxygen consumption by most of the media (Table 1). This indicated that superoxide radicals were formed during the autoxidation.

When ingredients of Trypticase soy broth (BBL) were autoclaved in various combinations and exposed to atmospheric oxygen, autoxidation and hydrogen peroxide accumulation were observed only in media in which both phosphate and glucose were present. A solution of phosphate and glucose autoxidized when it had been heated to 120°C for at least 5 min (Fig. 1) and the pH of the solution was higher than 6.5 (Fig. 2). The rate of autoxidation was increased with increasing heating time (Fig. 1), pH (Fig. 2), and concentration of glucose (Fig. 3) and phosphate (Fig. 4). A glucose solution heated in acetate, tris(hydroxymethyl)aminomethane (Tris)-maleate, or Tris-hydrochloride buffer in the pH range 5 to 8 only autoxidized in the Tris-hydrochloride buffers at a pH higher than 7.0. The rate of autoxidation was much slower in the Tris-hydrochloride buffer than in the phosphate buffer (Fig. 2).

Of other substances heated in potassium phosphate buffer, only α-hydroxycarbonyl compounds such as reducing sugars, glyceraldehyde, dihydroxyacetone, and glycolaldehyde autoxidized with accumulation of hydrogen peroxide (Table 2). A solution of the dicarbonyl compound glyoxal autoxidized at a slow rate (Table 2). When autoclaved in a water solution, none of these compounds gave autoxidizing products. A solution of thioglycolate in phosphate buffer also autoxidized, but no hydrogen peroxide was detected in the solution (Table 2). Nonreducing carbohydrates and a number of other hydroxyl and carbonyl compounds did not autoxidize when heated in phosphate buffer (Table 2).

Addition of SOD to an autoclaved solution of

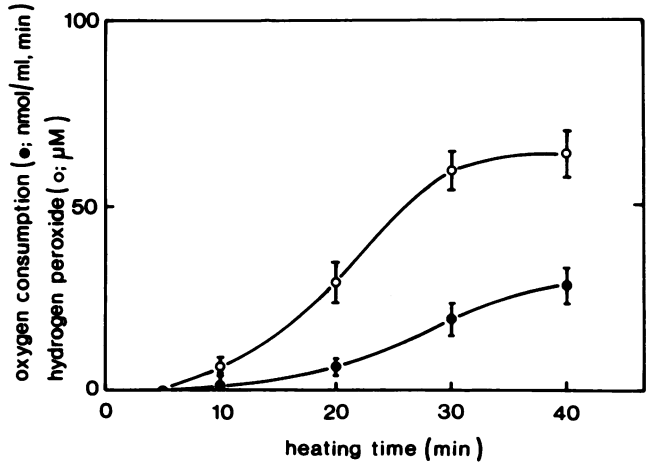

FIG. 1. *Autoxidation of 25 mM glucose in 0.25 M potassium phosphate buffer (pH 7.4), heated at 120°C for various times. The solutions were heated in double strength. Means ± standard deviation of four experiments are given.*

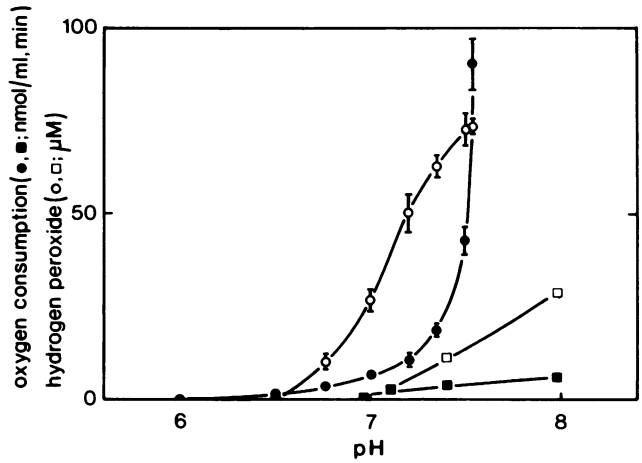

FIG. 2. *Autoxidation of 25 mM glucose in 0.25 M potassium phosphate buffer or in 0.25 M Tris-hydrochloride buffer at various pH values. The solutions were heated in double strength for 30 min at 120°C. Rate of oxygen consumption in phosphate buffer (●) and Tris-hydrochloride buffer (■) and the concentration of hydrogen peroxide accumulated in phosphate buffer (○) and in Tris-hydrochloride buffer (□) are shown. Means ± standard deviation of four experiments in phosphate buffer and one experiment in Tris-hydrochloride buffer are given.*

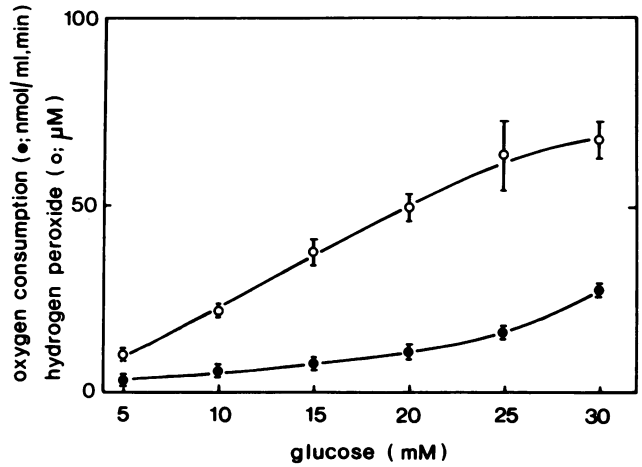

FIG. 3. *Autoxidation of various concentrations of glucose in 0.25 M potassium phosphate buffer (pH 7.4). The solutions were heated in double strength for 30 min at 120°C. Means ± standard deviation of four experiments are given.*

glucose in phosphate buffer decreased the rate of oxygen consumption by 70% (Fig. 5). This indicated that superoxide radicals were generated in the reaction and served as chain-propagating species. Addition of catalase to the reaction mixture also decreased the rate of oxygen consumption (Fig. 5). However, the oxygen concentration in the reaction mixture after 8 min was not significantly different whether catalase was added at the start of the reaction or at 8 min (Fig. 5). This indicated that hydrogen peroxide did not catalyze the reaction to a significant degree. It was not possible to inhibit the autoxidation by chelating agents such as 8-hydroxyquinoline (Table 3), *o*-phenanthroline, ethylene-diaminetetraacetic acid disodium salt, diethylenetriaminepentaacetic acid, citric acid, and 2,2'-bipyridyl in 1 mM concentration. Transitional metal ions were powerful catalysts of the autoxidation (Table 3). The absence of an effect of metal ion chelating agents indicated, however, that transitional metal ions were not necessary for the reaction to occur in an autoclaved solution of glucose and phosphate.

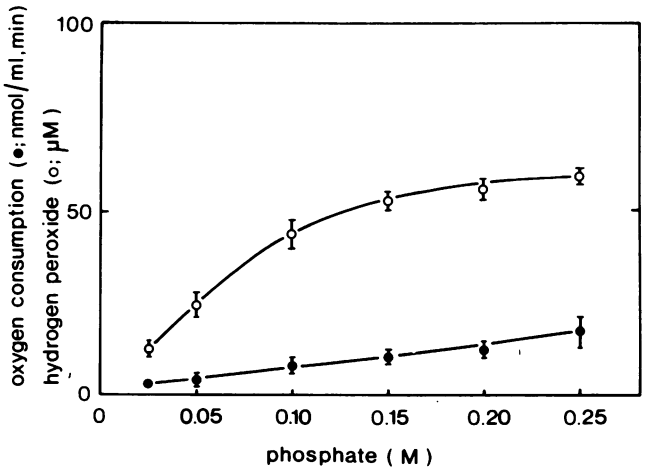

FIG. 4. *Autoxidation of 25 mM glucose in various concentrations of a potassium phosphate buffer (pH 7.4). The solutions were heated in double strength for 30 min at 120°C. Means ± standard deviation of four experiments are given.*

TABLE 2. *Autoxidation rate and hydrogen peroxide accumulation in autoclaved solutions of various compounds in 0.25 M potassium phosphate buffer, pH 7.4*[a]

Compound (27.5 mM)	Autoxidation rate (nmol of O_2 consumed/ml per min)	H_2O_2 accumulation (μM)
Glucose	20	60
Fructose	60	150
Maltose	40	118
L-Glyceraldehyde	146	56
1,3-Dihydroxyacetone	121	64
Glyoxal	1.2	15
Glycolaldehyde	20	48
Sodium thioglycolate	6.4	0

[a] The solutions were heated in double strength for 30 min at 120°C. The following compounds did not give autoxidizing solutions: mannitol, sorbitol, sucrose, α-methyl-glycoside, sodium pyruvate, sodium lactate, sodium formate, sodium citrate, glyoxylic acid, cystine, acetaldehyde, acetaldol, and acetone.

DISCUSSION

It is an old observation that autoclaving phosphate and glucose together in culture media makes the media unsuitable for growth of many microorganisms (2, 10, 20, 24), but the toxic products formed in the media have never been identified. The present study showed that such a treatment of phosphate and glucose at neutral or alkaline pH resulted in products which rapidly autoxidized with the formation of superoxide radicals and hydrogen peroxide. The toxicity of hydrogen peroxide in culture media is well established (1, 9, 13, 17, 18, 28). A direct toxic effect of superoxide radicals formed in the autoxidation of culture media (13) or in the autoxidation of dialuric acid in culture media (9) has not been demonstrated, but the involvement of superoxide radicals in the bactericidal effect of oxygen has been shown in studies where superoxide radicals have been generated by streptonigrin (14) or by photochemically reduced flavins (16). Although superoxide radicals may not be directly toxic in culture media, they may play an important role as chain-propagating species in the autoxidation of the media, and the autoxidation could result in toxic concentration of hydrogen peroxide (13). With SOD present in the media, the autoxidation rate decreases, the concentration of hydrogen peroxide accumulated in the media is reduced, and the media have a lower toxic effect (13). It should also be remembered, however, that autoclaving glucose and phosphate with the medium could be necessary for rapid and consistent growth of many lactic acid bacteria (8, 27, 29, 30, 33, 34). In the autoxidation of a complex culture medium, many types of reactive species could be formed. The mechanism of the bactericidal effect as well as of the growth-promoting effect could be multifactorial. The beneficial effect of catalase for the survival and growth of many bacteria in various culture media (5, 6, 13, 17, 18, 28) indicates that hydrogen peroxide is an important mediator of the toxic effect by media exposed to atmospheric oxygen. However, hydrogen peroxide by itself may not be the ultimate toxic product in these media. Hydrogen peroxide may be reduced to the highly reactive hydroxyl radical (15) or may react with, e.g., glyoxal, glycolaldehyde (32), and histidine (31) to form adducts

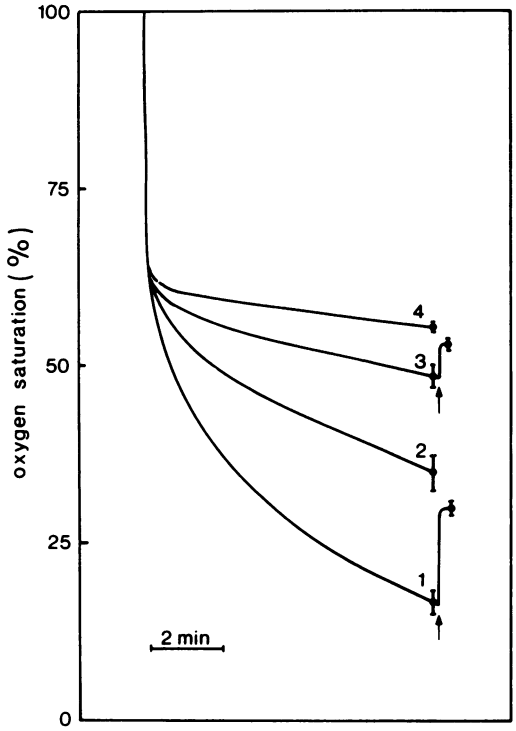

FIG. 5. *Autoxidation of 25 mM glucose in 0.25 M potassium phosphate buffer (pH 7.4) in the presence of catalase and SOD. The solutions were heated in double strength for 30 min at 120°C. Line 1 demonstrates the oxygen consumption as measured with a Clark oxygen electrode at 25°C in a solution made up of 4 ml of water, saturated with atmospheric oxygen, and 4 ml of the anaerobic autoclaved solution. Catalase (375 μg in 15 μl) was added as shown by arrows. Line 2 shows the oxygen consumption when 375 μg of catalase was added to the water at the start of the experiment. Line 3 shows the effect of an addition of 167 μg of SOD and line 4, an addition of 375 μg of catalase + 167 μg of SOD at the start of the experiment. Means ± standard deviation of three experiments are given.*

that are more toxic than the individual compounds alone. Some of these adducts may be decomposed by catalase (26, 35). The growth-promoting effect of media in which glucose and phosphate have been autoclaved has been ascribed to the formation of various substances during the heating. Pyruvic acid could substitute for autoclaved glucose in the media, and is effective as a growth-promoting factor for *Streptococcus salivarius* (33) and *Lactobacillus bulgaricus* (34). *N*-D-glucosylglycine has a similar effect for the growth of *L. gayoni* (30). With an organism such as *S. faecalis*, autoclaving the medium can be dispensed with if ascorbic acid, cysteine, or other reducing agents are added to the medium (29). The present study clearly demonstrated that heating glucose and phosphate together resulted in the formation of highly effective reducing products. These products should be able to provide a reduced environment for the growth of many bacteria.

TABLE 3. *Autoxidation rate and hydrogen peroxide accumulation in autoclaved glucose exposed to oxygen in the presence of various metal ions and 8-hydroxyquinoline*[a]

Metal ion (concn; μM) 8-HQ in reaction mixture	Autoxidation rate (nmol of O_2 consumed/ml per min)	H_2O_2 accumulated (μM)
Fe^{2+} (100)	59	20
Fe^{2+} (10)	30	63
Fe^{2+} (10) + 8-HQ	19	70
Cu^{2+} (10)	83	80
Cu^{2+} (10) + 8-HQ	19	59
Mn^{2+} (100)	50	75
Mn^{2+} (10)	33	71
Mn^{2+} (10) + 8-HQ	29	79
Mg^{2+} (100)	26	67
Mg^{2+} (10)	21	62
Mg^{2+} (10) + 8-HQ	20	75
None	20	60
8-HQ	20	63

[a] Autoclaved 27.5 mM glucose in 0.25 M potassium phosphate buffer (pH 7.4) was exposed to oxygen in the presence of various concentrations of metal ions and 1 mM 8-hydroxyquinoline (8-HQ). The solutions were heated in double strength for 30 min at 120°C.

Hydrogen peroxide appeared to be the most important toxic product in the autoxidation of the media, but hydrogen peroxide in any significant concentration was not detected in any of the five thioglycolate media tested. Brewer thioglycolate medium (Difco) autoxidized rapidly and was highly toxic, while Clausen medium (Oxoid) was not toxic. The latter medium contains thioglycolate and dithionite and has excellent growth-promoting properties (7). Two media, fluid thioglycolate medium (BBL) and Brewer thioglycolate medium (Oxoid), have similar composition, but the Oxoid medium was much more toxic for *P. anaerobius* VPI 4330-1 than the BBL medium. These differences in toxicity could not be explained from the results of the present study, and obviously other mechanisms for the bactericidal effect of autoxidizing broth media have yet to be identified. Work in progress shows that autoxidizing cysteine has a potent toxic effect on *P. anaerobius* VPI 4330-1. The most toxic medium included in this study was actinomyces broth (BBL), and that broth

was also the only medium that contained cysteine. The bactericidal effect of actinomyces broth may therefore be a combined effect of products formed during the autoxidation of cysteine and of sugars autoclaved in the presence of phosphate.

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