JOURNAL OF BACTERIOLOGY, Oct. 1973, p. 183-191
Vol. 116, No. 1

Printed in U.S.A.

Induction of Unbalanced Growth and Death of *Streptococcus sanguis* by Oxygen

ROSELYN J. EISENBERG

Department of Microbiology, School of Dental Medicine, University of Pennsylvania and The Center for Oral Health Research, Philadelphia, Pennsylvania 19174

Received for publication 26 April 1973

Streptococcus sanguis has normal streptococcal morphology when grown in a nitrogen atmosphere but undergoes gross morphological alterations when grown in the presence of oxygen (O cells). Studies were made of the relationship between the development of pleomorphism and the loss of viability which accompanied it in O cultures of *S. sanguis*. The development of pleomorphism appears to involve two events. The first step, or triggering event, occurs in log-phase cultures in the presence of oxygen. The second step, or manifestation of pleomorphism, occurs at stationary phase in the presence or absence of oxygen. The loss of viability appears to be related both to the length of time the culture is exposed to oxygen as well as to a specific event which occurs at the transition from log to stationary phase. Oxygen also induced a state of unbalanced growth in *S. sanguis* in which the proportions of deoxyribonucleic acid, ribonucleic acid, and protein synthesized were altered. The relationship of these alterations to the development of pleomorphism is discussed.

Streptococcus sanguis undergoes gross morphological changes when grown in the presence of oxygen (O cells). However, normal morphology is maintained when the cells are grown in an atmosphere of nitrogen (N cells) or when O cells are grown in the presence of catalase (B. Rosan and R. J. Eisenberg, Arch. Oral Biol., in press). This latter observation suggests that a peroxide is involved in the development of pleomorphism in *S. sanguis*.

Many temperature-sensitive and other conditional bacterial mutants have been isolated that undergo gross morphological changes at the nonpermissive condition (8, 13, 15-17, 21). When these changes are lethal, they are often associated with other physiological alterations such as cessation of deoxyribonucleic acid (DNA) synthesis. We noted that the morphological changes occurring in *S. sanguis* were accompanied by a significant loss of viability (B. Rosan and R. J. Eisenberg, Arch. Oral Biol., in press). This observation suggested that oxygen might affect several physiological properties of *S. sanguis*. However, the two phenomena might be caused independently by different oxygen-sensitive mechanisms. We undertook studies to determine the relationship between development of pleomorphism and loss of viability in O cultures of *S. sanguis*. We also studied the relationship of pleomorphism to other changes in growth and macromolecular synthesis in *S. sanguis*.

MATERIALS AND METHODS

Bacterial strain. Strain G9B of *S. sanguis* used in these studies was isolated from dental plaque on Mitis-Salivarius agar (Difco). The strain was identified as *S. sanguis* by criteria described by DeStoppelaar (5) and Carlsson (3).

Culture technique. The organisms were grown in brain heart infusion broth (BHI) (Difco) at 37 C. Inocula were taken from broth cultures that had been stored at −100 C and thawed only once. Fresh inocula were prepared by defrosting 1 ml of bacteria (5×10^8 cells), diluting with prewarmed medium to 5 ml, and incubating for 2 to 3 h. This culture was then further diluted into 100 ml of BHI broth and incubated overnight at 37 C. For experiments, this culture was diluted 20-fold into fresh, prewarmed medium.

A mixture of 95% N_2 plus 5% CO_2 (for N cells) or 95% O_2 plus 5% CO_2 (for O cells) was bubbled through the medium for 1 h prior to inoculation and continued throughout the course of growth.

Quantitation of growth. Turbidity was measured in a Klett-Summerson colorimeter equipped with a no. 66 filter. For quantitating total cell numbers, culture samples were chilled to 0 C, fixed with 5% formaldehyde, and counted in triplicate within 1 to 2 days. There was no detectable change in cell number or clumping of cells in this period of time. After 3 to 4

days, however, chains tended to shorten. When necessary, further dilutions were made with BHI broth, and approximately 500 cells were counted in a Petroff-Hausser chamber by using a Zeiss phase-contrast microscope fitted with a Zeiss Neofluar 100× oil immersion objective. Each cell, whether free or in a chain, was tallied as a single cell. Cells with obviously incomplete cross walls were tallied as one cell. The number of colony-forming units (CFU) per milliliter of the culture was determined by spreading appropriate dilutions in quadruplicate on BHI agar and incubating the plates at 37 C for 1 to 2 days. Care was taken to mix the culture samples and dilutions vigorously to break up chains. Using this method, the standard error of the mean of quadruplicate counts in three separate experiments was ±3 to 8%.

Phase-contrast photomicroscopy. Culture samples were fixed with 5% formaldehyde, placed on slides (1 by 3 inches) previously overlaid with a thin film of 1.5% agar (Difco, purified), fitted with a cover slip, and examined in a Zeiss phase-contrast microscope under oil immersion. The film used was Kodachrome IIA.

Isotope incorporation. For each isotope, 200 ml of BHI broth containing the appropriate labeled precursor were inoculated with 10 ml of an overnight seed culture. N and O cultures were incubated as previously described. The following isotopes and final concentrations were used to measure macromolecular synthesis: for protein, L-leucine-*4,5*^3H, 1.0 Ci/ml (sp act 5 Ci/mmol); for ribonucleic acid-(RNA), uridine-*5*-^3H, 0.5 Ci/ml (sp act 28.2 Ci/mmol); and for DNA, thymidine-*methyl*-^3H, 1.0 Ci/ml (sp act 18 Ci/mmol). All isotopes were purchased from New England Nuclear Corp. The incorporation of tritium-labeled precursors into acid-precipitable material was determined by the filter-paper disk method of Bollum (1). The disks were placed in vials containing 10 ml of Liqifluor (New England Nuclear Corp.) and counted in an Intertechnique liquid scintillation spectrometer.

RESULTS

Effects of oxygen on the growth and morphology of S. sanguis. To study the effects of oxygen on the growth and morphology of *S. sanguis* in detail, we examined N and O cultures at intervals during growth for microscopic appearance (Fig. 1), turbidity (Fig. 2a), CFU (Fig. 2b), and total cell number (Fig. 2c). Synthesis of DNA, RNA, and protein (Fig. 3) were measured in separate experiments by adding the appropriate radioactively labeled precursor to the culture at the time of inoculation and measuring incorporation of the label into trichloroacetic acid-precipitable material. Each of the labeled cultures was monitored turbidimetrically and showed essentially the same growth kinetics as in Fig. 2a.

(i) Properties of N cells. The turbidity of the N culture (Fig. 2a) rose exponentially for 3 h and then leveled off by 4 h at a maximum of approximately 140 Klett units. Synthesis of RNA and protein (Fig. 3b, c) followed a similar pattern of increase. By 4 h after inoculation, incorporation of ^3H-uridine or ^3H-leucine into acid-precipitable material had reached a maximum. The mass doubling of the N culture during log phase was approximately 40 min when measured turbidimetrically, but the generation time was approximately 80 min when measured by total cell number or CFU (Fig. 2b, 2c). These data indicate that, during the exponential phase, the growth rate of the N culture was faster than the rate of cell division. Phase-contrast micrographs of the N culture taken after 1.5 and 3 h of growth (Fig. 1a, b) show that long chains of cells developed during this time. Many cells in these chains had obviously incomplete septa and were tallied as one cell rather than two in determining total cell counts. Even though samples were mixed vigorously before being plated, the apparent number of CFU could have been lowered by the presence of chains and cells with incomplete septa. Thus, the observed difference between growth rate (Fig. 2a) and the rate of cell division (Fig. 2b, 2c) may, in part, reflect chain formation and incomplete septation of cells in log phase N cultures.

After 3 to 4 h, turbidity (Fig. 2a), RNA (Fig. 3b), and protein synthesis (Fig. 3c) had reached maximal levels (stationary phase). However, DNA synthesis (Fig. 3a) and cell division (CFU, Fig. 2b, total cell number, Fig. 2c) continued for 2 to 3 h longer. Microscope examination of N cultures after 5 and 24 h of growth (Fig. 1c, d) indicated that chains contained fewer numbers of cells and that most of the cells appeared to have complete septa. In 24-h cultures, the cells were seen mostly in pairs. Thus, completion of septa, some DNA synthesis, and unchaining took place in the N culture after other aspects of growth had stopped.

(ii) Properties of O cells. The kinetics of growth and macromolecular synthesis in O cells followed a different pattern from that observed for N cells. The turbidity of the O culture rose more slowly and reached a maximum of only 65 Klett units. During log phase (the first 2 h of growth), the mass doubling time of the O culture, calculated turbidimetrically (Fig. 2a) was approximately 55 min. However, the rates of protein and RNA synthesis (Fig. 3b, c) in the O culture were the same as in the N culture for the first 2 h of growth. The discrepancy in the mass doubling time of the two cultures cannot be attributed to differences in the exponential rates of synthesis of these macromolecules.

After 2 h, RNA synthesis stopped abruptly in the O culture, while protein synthesis continued

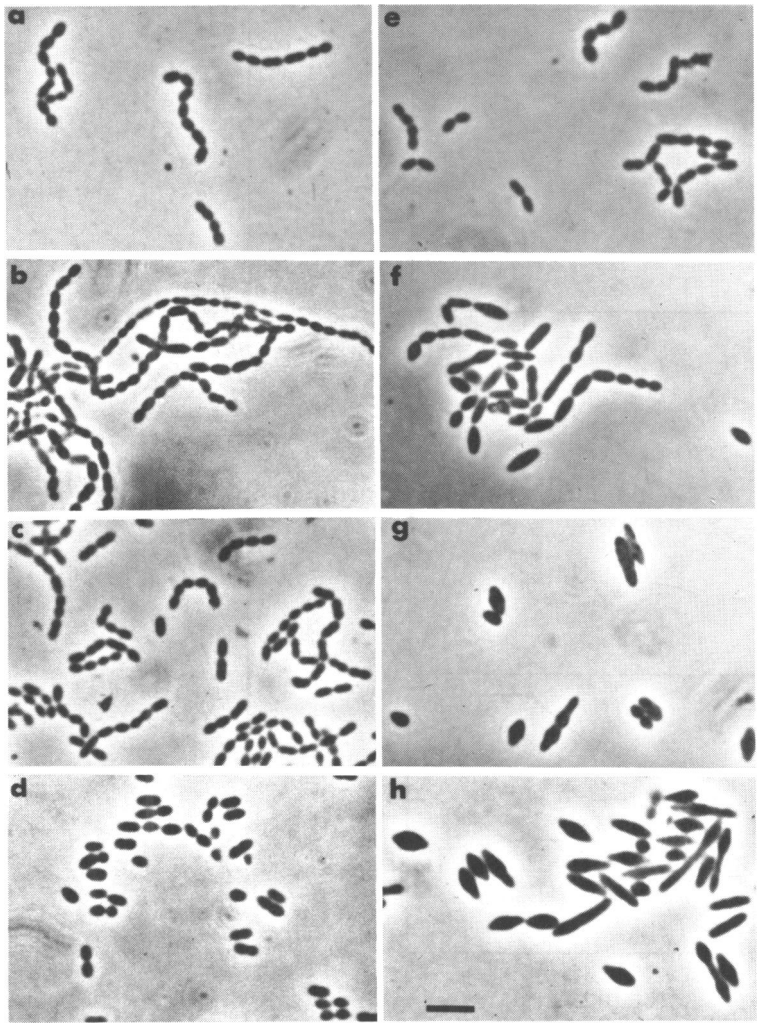

FIG. 1. *Phase-contrast micrographs of N and O cells of Streptococcus sanguis. (a–d), N cells after 1.5, 3, 5, and 24 h of growth; (e–h), O cells after 1.5, 3, 5, and 24 h of growth. Details of growth in legend to Fig. 2. Marker bar, 5 μm.*

for approximately 2 to 3 h longer at a decreasing rate. At stationary phase, the O culture incorporated 50% as much uridine into RNA as did the N culture. There was a similar difference in the total amount of leucine incorporated into protein. These data agree well with the twofold difference in maximal turbidity of the two cultures. A small burst in the number of CFU occurred between 2 and 3 h, and after that time DNA synthesis leveled off and the number of CFU did not increase. The increase in turbidity that occured between 2 and 3 h in the absence of a concomitant increase in cell number suggests that O cells increased significantly in size. This conclusion was confirmed by phase-contrast microscopy. Log-phase O cells were quite similar in appearance to log-phase N cells during the first 2 to 3 h of growth (compare Fig. 1a with Fig. 1e). Morphological abnormalities such as enlargement and elongation were noticeable in some cells at 3 h or the end of log phase (Fig. 1f). By 5 h, (Fig. 1g) there was a noticeable increase in the size of O cells, as well as gross changes in cell shape. It is of some interest that after 3 h very few chains of cells were observed in the O culture. By 5 h almost all of the cells occurred singly. The increase in CFU that occurred in the culture at 3 h (Fig. 2b) may have been due to the unchaining of cells that appeared to accompany the onset of pleomorphism.

A puzzling observation was that DNA synthesis took place at a slightly faster rate in the O

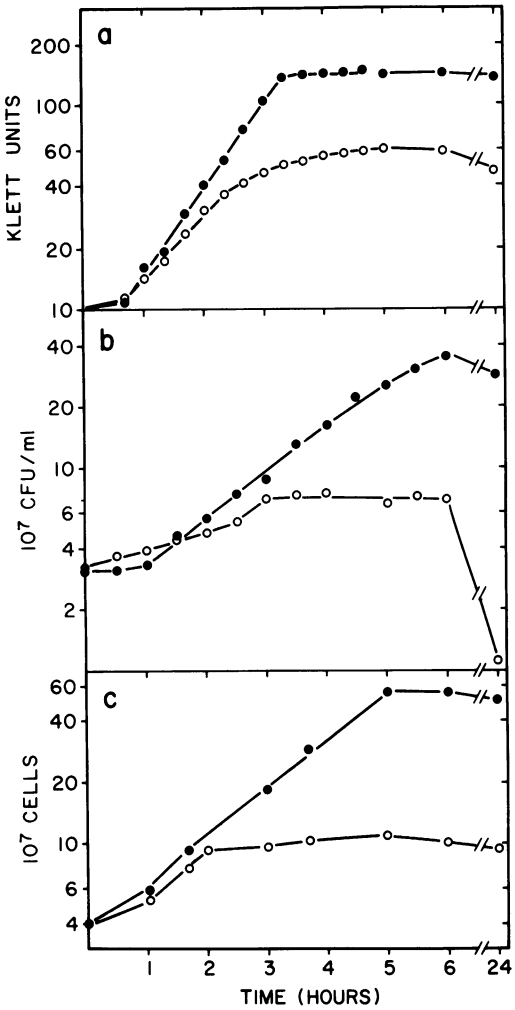

FIG. 2. *Growth kinetics of N and O cells of Streptococcus sanguis. For this experiment, two flasks each containing 500 ml of BHI broth were inoculated with 25 ml of an overnight culture of S. sanguis. The N culture (●) was incubated in an atmosphere of 95% N_2 plus 5% CO_2 at 37 C. The O culture (○) was incubated in an atmosphere of 95% O_2 plus 5% CO_2 at 37 C. At 20-min intervals, 10-ml samples were removed and assayed for (a) turbidity (b) total cell number per milliliter, and (c) colony-forming units per milliliter. At 1-h intervals 2-ml samples were fixed with 5% formaldehyde and chilled to 0 C for microscopy.*

culture than in the N culture and leveled off more abruptly. The total amount of thymidine incorporated per milliliter was nearly the same for the two cultures. Since the O culture contained only 20% as many cells per ml as the N culture (Fig. 2b, c) it appears that each O cell contained as much as five times more DNA

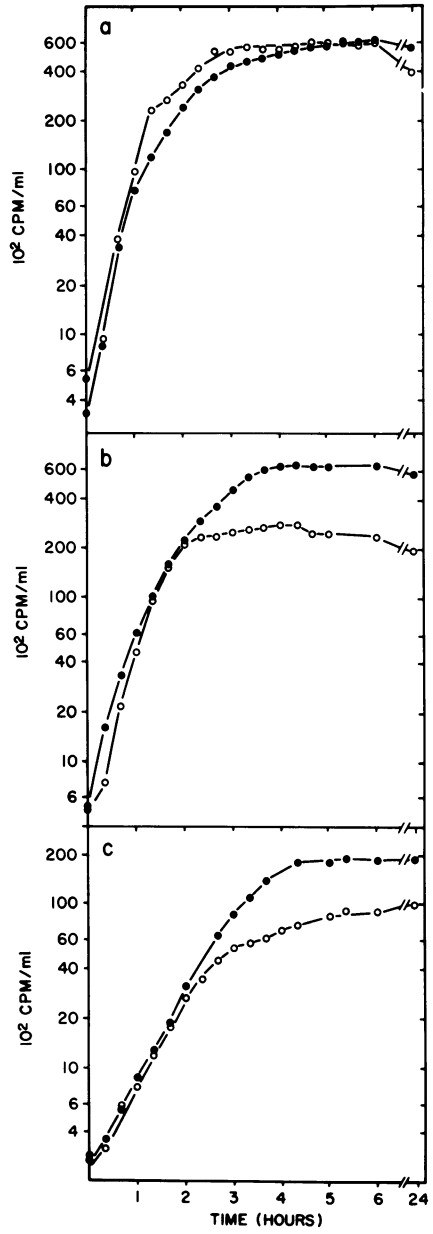

FIG. 3. *Kinetics of macromolecule synthesis in Streptococcus sanguis. The appropriate isotope was added to each flask of 200 ml of BHI prior to inoculation with a labeled overnight culture of S. sanguis. The N culture (●) was incubated in an atmosphere of 95% N_2 plus 5% CO_2 at 37 C. The O culture (○) was incubated in an atmosphere of 95% O_2 plus 5% CO_2 at 37 C. (a) Incorporation of ^{3}H-thymidine, 10^2 counts per min per ml of culture; b, incorporation of ^{3}H-uridine, 10^2 counts per min per ml of culture; c, incorporation of ^{3}H-leucine, 10^2 counts per min per ml of culture.*

than each N cell. This conclusion was confirmed by colorimetric assay (2) of the DNA content of N and O cells (R. Eisenberg, unpublished results).

If O cells were merely larger than N cells, the ratio of one macromolecule to another would remain constant. The fact that different proportions of DNA, RNA, and protein were synthesized, indicates that oxygen induced a state of unbalanced growth in *S. sanguis*. It is not unreasonable that conditions which produced this state of unbalanced growth also led to the development of pleomorphism. Since unbalanced growth often leads to cell death (4, 19), it is not surprising that exposure to oxygen also caused significant losses in viability of *S. sanguis*. Experiments were undertaken to determine (i) whether the effects of oxygen on viability and morphology took place during a particular phase of growth or were related to the length of time the culture was exposed to oxygen and (ii) whether the change in morphology and loss of viability were related to the same events or occurred independently.

Dependence of pleomorphism and loss of viability upon the growth phase. *S. sanguis* was grown in an atmosphere of 95% nitrogen plus 5% CO_2 and at 30-min intervals; samples were removed and incubated in an atmosphere of 95% oxygen plus 5% CO_2 for the remainder of a 24-h period. Table 1 shows when visible morphological changes occurred in cells switched from nitrogen to oxygen. Five of the cultures that became pleomorphic (samples 1–5) reached a much lower turbidity than the control N culture (sample 12). Sample 6, switched at 2.5 h, appears to represent an intermediate. The maximal turbidity of this culture was 87% of the control, and only a fraction of the cells appeared to be pleomorphic. Moreover, chains were abundant in this culture, and individual chains contained both pleomorphic and coccal forms. When cultures were switched later than 2.5 h (samples 7–11), turbidity approached the maximum reached by the control N culture and the cells retained their coccal shape. These results indicate that the development of pleomorphism depended upon exposure of *S. sanguis* to oxygen during the first 2 h of growth (log phase). After 2.5 h, the N culture was refractive to the effects of oxygen. By this time, growth was 90% complete. Thus, the factor(s) which caused pleomorphism was generated by an actively growing culture.

In contrast, the number of CFU was reduced in every culture switched from nitrogen to oxygen, the greatest reductions occurring in cultures exposed to oxygen for the longest time. These results indicate that the loss of viability depends upon exposure time to oxygen. Thus, although the morphological changes took place only in cultures showing an increase in mass, killing continued to occur after growth stopped. However, there was a 10-fold difference in the number of CFU found in sample 9 (switched at 4 h) and the number found in sample 10 (switched at 4.5 h). Therefore, the loss of viability may also be due in part to a growth phase-dependent phenomenon which occurs after the onset of stationary phase. This suggests that at least some of the events involved in causing cell death occur later in the growth of *S.*

TABLE 1. *Growth of S. sanguis cultures switched from nitrogen to oxygen at various times after inoculation*[a]

Sample no.	Time switched (h after inoculation)	Turbidity at 5 h		Viability after 24 h		Morphology after 24 h	
		Klett units	Percentage of sample 12	10^8 CFU/ml	Percentage of sample 12	Shape of cells	Arrangement of cells
1	0.0	64	43	0.03	<0.1	Pleomorphic	Single
2	0.5	61	41	0.08	<0.1	Pleomorphic	Single
3	1.0	62	41	0.13	<0.1	Pleomorphic	Single
4	1.5	73	49	0.2	<0.1	Pleomorphic	Single
5	2.0	103	69	0.40	0.1	Pleomorphic	Single
6	2.5	130	87	0.80	0.3	Pleomorphic	Chains
7	3.0	148	99	1.1	0.4	Cocci	Chains
8	3.5	150	100	2.2	0.8	Cocci	Chains
9	4.0	144	96	10	3.7	Cocci	Chains
10	4.5	143	95	100	37	Cocci	Chains
11	5.0	142	99	120	44	Cocci	Pairs, chains
12	24.0	150	100	270	100	Cocci	Pairs

[a] In this experiment, 500 ml of BHI broth was inoculated with 25 ml of an overnight N culture and grown in a nitrogen atmosphere at 37 C (sample 12). At various times after inoculation, 40 ml was removed to another flask and incubated in an oxygen atmosphere at 37 C for the remainder of the 24 h.

sanguis than events related to the development of pleomorphism. Moreover, the events involved in cell death appear to occur independently of visible morphological changes. This latter conclusion is based on the observation that samples 7 to 9 in Table 1 contained no pleomorphic forms but contained fewer than 5% as many CFU as the N control at 24 h. Because of the temporal sequence of these events, it is impossible to state whether there is a cause-effect relationship between pleomorphism and loss of viability.

Effect of a 1-h oxygen pulse on S. sanguis. The previous experiment suggests that pleomorphism and cell death are caused by separate and independent events that occur in the presence of oxygen at different and specific times during the growth of *S. sanguis*. Thus, a culture exposed to oxygen for a specific time or pulse period might develop one of these changes and not the other. To test this hypothesis, a culture of *S. sanguis* was grown in a nitrogen atmosphere, and at various intervals samples were removed, pulsed with oxygen for 1 h, and then reincubated in nitrogen (Fig. 4). Table 2 summarizes the effects of the oxygen pulse on the morphology, maximal turbidity, and viability of each sample. By phase-contrast microscopy, samples 1, 4, 5, 6, and 7 had the same morphology as the main culture (sample 8) after 5 or 24 h. However, cultures 2 and 3, pulsed during the mid-log phase (Fig. 4) contained pleomorphic cells after 5 h of total growth. At 24 h, the cells

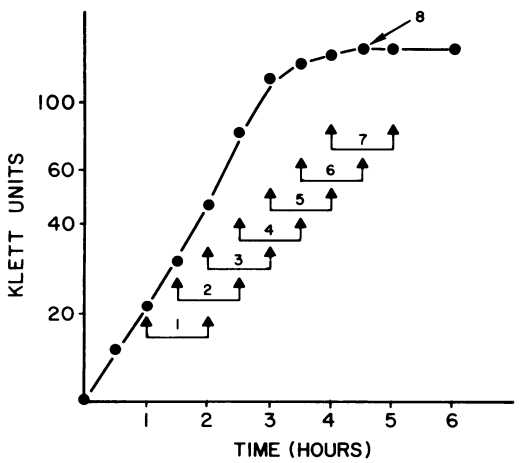

FIG. 4. *Removal of 40-ml samples from 400 ml of an N culture (number 8) of Streptococcus sanguis for a 1 h "pulse" of oxygen. Samples 1 to 7 were incubated in an atmosphere of 95% O_2 plus 5% CO_2 for the periods bracketed by the arrows (↑ ↑) and then returned to an atmosphere of 95% N_2 plus 5% CO_2. All cultures were incubated at 37 C.*

in these two cultures regained their coccal shape but occurred in long chains rather than in pairs. The maximal turbidity of these two cultures was lower than that reached by any of the other cultures. Thus, the oxygen pulse appeared to have a specific effect on the turbidity and morphology of *S. sanguis* if it was given during a restricted portion of the log phase. Although the two cultures that became pleomorphic contained fewer CFU than the main culture, the number was not significantly lower than for the cultures that did not become pleomorphic. Sample 4 which was pulsed at late log-early stationary phase suffered a significant loss of viability but did not undergo any noticeable morphological changes.

Several conclusions can be drawn from this experiment. (i) The morphological changes appear to have been triggered by events requiring growth of *S. sanguis* in an oxygen atmosphere during mid-log phase. This confirms the conclusion drawn from the previous experiment. (ii) Pleomorphism was manifested several hours after the pulse. (iii) The morphological changes induced by brief periods of exposure to oxygen were reversible. (iv) The oxygen pulse had a very pronounced effect on the viability of a culture in late log-stationary phase and less effect on cultures of other ages. (v) Pleomorphism and cell death occurred independently and appear to be triggered by separate events that occurred at different times during growth.

Relationship of pleomorphism to stationary phase. An experiment was performed to determine why the morphological changes occurred several hours after the oxygen pulse. It could be argued that the changes merely required several hours to manifest themselves. However, since the morphological changes took place just prior to the onset of stationary phase, it is possible that events which occurred specifically at this time were also involved in pleomorphism. An O culture was maintained in log phase for longer than 5 h, the time it usually takes for most cells in the culture to become noticeably pleomorphic. The culture was grown in an oxygen atmosphere to mid-log phase (culture A) then diluted threefold with fresh, prewarmed medium. This diluted culture (B) was again diluted threefold when it reached mid-log phase. A total of three dilutions were made over a period of 5 h. Half of the last culture (D) was removed and placed in a nitrogen atmosphere (culture E) at late log phase (7.5 h). The other half of this culture was left to continue growth in an oxygen atmosphere. Figure 5 shows the growth kinetics of each of the diluted cultures (A–D). Although

TABLE 2. *Effects of a 1-h oxygen pulse on the morphology and growth of Streptococcus sanguis*

Sample no.	Time of pulse (h after inoculation	Maximal turbidity		Viability after 24 h		Morphology of cells	
		Klett units	Percentage of sample 8[a]	10^6 CFU/ml	Percentage of sample 8[a]	3-5 h after pulse	24 h after inoculation
1	1-2	125	81	160	53	Cocci, chains	Cocci, pairs
2	2-3	111	72	140	47	Pleomorphic, single	Cocci, chains
3	3-4	111	72	90	30	Pleomorphic, single	Cocci, chains
4	4-5	137	88	14	5	Cocci, chains	Cocci, pairs
5	5-6	150	98	82	27	Cocci, chains	Cocci, pairs
6	6-7	153	99	190	63	Cocci, chains	Cocci, pairs
7	7-8	157	100	230	78	Cocci, chains	Cocci, pairs
8[a]		155	101	300	100	Cocci, chains	Cocci, pairs

[a] Main culture, containing 400 ml of BHI broth, inoculated with 20 ml of an overnight N culture and incubated in a nitrogen atmosphere at 37 C. At the times indicated in Fig. 4, 40 ml was removed to a separate flask, grown for 1 h in an oxygen atmosphere at 37 C, and then switched to a nitrogen atmosphere for the remainder of the experiment.

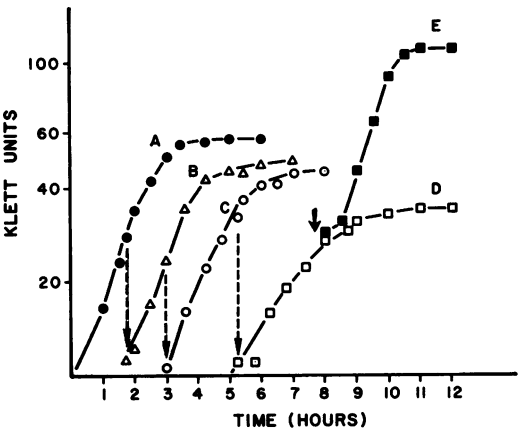

FIG. 5. *Maintenance of Streptococcus sanguis in an oxygen atmosphere. In this experiment, 200 ml of BHI broth was inoculated with 10 ml of an overnight N culture and incubated in an atmosphere of 95% O_2 plus 5% CO_2. When the culture (●) reached log phase (indicated by dashed arrow (↓)), 50 ml was removed to a flask containing 150 ml of prewarmed, pregassed medium (culture B, △). This procedure was repeated twice more at the times indicated by dashed arrows (↓) for cultures C (○) and D (□). When culture D reached late log phase (solid arrow (↓)), 100 ml was removed to a separate flask and incubated in an atmosphere of 95% N_2 plus 5% CO_2 (culture E, ■). All cultures were incubated at 37 C.*

the growth rate dropped somewhat at each subculture, no morphological changes were detected by phase microscopy until the subcultures entered stationary phase. Cultures A–D showed pleomorphic forms 2 to 3 h after stationary phase began. Thus, in culture D, the actual time when pleomorphism was first detectable was 11 h after inoculation of the original culture.

This experiment indicates that *S. sanguis* cells could be exposed to oxygen for long periods of time with no evidence of pleomorphism, provided that the culture remained in log phase. Therefore, the development of pleomorphism in *S. sanguis* appears to require two sets of events, one which is dependent upon oxygen and occurs during log phase, and another which occurs independently of oxygen as the culture enters stationary phase.

Pleomorphism was a transient event in subculture E which entered stationary phase in the presence of nitrogen. The pleomorphic forms which were observed in this culture at early stationary phase were not found after 24 h. Cultures A to D remained pleomorphic at 24 h. However, culture E was switched to nitrogen prior to the time when viability would be affected. This culture contained 2.3×10^8 CFU at 24 h, whereas cultures A to D contained less than 1×10^6 CFU. Thus, the cells in cultures A to D were fixed in a pleomorphic form, but the cells in culture E resumed growth and reverted to a coccal form.

DISCUSSION

The present studies indicate that oxygen induced a state of unbalanced growth in *S. sanguis* characterized by gross morphological changes and cell death. When *S. sanguis* was grown in a nitrogen atmosphere, the mass doubling time was 50% of the division time during log phase. For several hours after the turbidity reached a maximum, cell division continued in this culture. These data indicate that, in N cells, cell division lags behind mass increase during the log phase and catches up during stationary phase. These observations are

similar to those made by Mitchison (12) who found that, in *S. fecalis*, septation constantly lags behind nuclear division. When *S. sanguis* was grown in the presence of oxygen, the turbidity of the culture continued to increase for several hours after cell division stopped. Assuming that turbidity is an accurate reflection of cell mass in the O culture, this observation suggests that O cells were considerably larger than N cells at stationary phase. Direct confirmation of this conclusion was made by phase-contrast microscopy. Differences in the size of some O cells were noticeable after 3 h of growth (end of log phase), and gross changes in morphology and size were apparent in most of the cells after 5 h (stationary phase). Thus in O cells, division was not able to catch up with mass increase as it did in stationary-phase N cells. Moreover, the effect of oxygen on morphology is a growth phase-dependent phenomenon involving two steps. The first step, or triggering event, occurred in log phase or actively growing cultures in the presence of oxygen, and the second step, the actual manifestation of pleomorphism, took place at stationary phase in the presence or absence of oxygen.

The extent to which oxygen affected the viability of *S. sanguis* depended upon the growth phase as well as duration of exposure. The longer a culture of *S. sanguis* was exposed to oxygen, the greater was the loss of viability. However, cultures exposed briefly to oxygen just at the transition from log to stationary phase suffered greater losses of viability than cultures pulsed with oxygen before or after this time. The specific event which caused this loss of viability occurred prior to the time when pleomorphism was manifested. Because of the temporal sequence of these events, it is impossible to state whether there is a cause-effect relationship between pleomorphism and loss of viability. Analysis of the biochemical events which are involved in these changes is required before this problem can be resolved. The evidence does suggest that pleomorphic cells are incapable of dividing unless they can resume normal coccal morphology. Therefore, long-term survival may be impossible for pleomorphic cells. This situation is analogous to that observed for many temperature-sensitive morphology and division mutants. At the non-permissive condition, these bacteria undergo morphological alterations and stop dividing. When they are returned to the permissive condition, growth is resumed and the cells also reassume their characteristic morphology (8, 11, 13, 15). Like *S. sanguis*, these bacteria die at the nonpermissive condition. It is usually assumed that cell death at the nonpermissive condition is causally related to pleomorphism (11). However, in the case of *S. sanguis*, conditions were found which separated oxygen-induced pleomorphism from oxygen-induced cell death. When stationary-phase N cultures were exposed to oxygen or when cultures were pulsed with oxygen at the beginning of stationary phase, viability decreased significantly, but the cells did not become pleomorphic.

The alteration in the pattern of macromolecular synthesis that occurred in O cells may offer some clues as to the nature of the biochemical lesion(s) that caused pleomorphism and cell death. Both events were preceded by an abrupt cessation of RNA synthesis. Although protein synthesis continued in the O culture for several hours longer, the rate was drastically reduced. Thus, the synthesis of specific proteins, such as those postulated to be involved in cell division (9, 13, 19), may have been altered or prevented by the absence of the necessary RNA. In this regard, it is of some interest that DNA synthesis was not drastically affected by oxygen. This suggests that cell division was blocked at a step which occurred after DNA synthesis. In this respect, the pleomorphic form of *S. sanguis* may resemble some of the septation mutants of *B. subtilis* and *E. coli* (11, 15, 16, 21). If this is the case, the proteins of O cells might differ qualitatively from those of N cells. Studies of the proteins made by the two cell types are now underway.

One of the proteins altered by oxygen might be an autolysin. It has been proposed that autolytic enzymes are involved in the unchaining of streptococci (6, 10) and other bacteria (7, 20). In N cells of *S. sanguis*, these enzymes were apparently inactive or latent (14) until the end of log phase when septation was completed and chains broke up. Although no gross morphological changes took place in log-phase O cells, the long chains that were characteristic of log-phase N cells were absent. Moreover, when pleomorphism was manifested in the presence of nitrogen, the cells eventually regained their coccal shape but occurred in long chains rather than pairs. These observations suggest that the normal autolytic process is disrupted by oxygen. Speculation about the mechanism of this disruption and its relation to pleomorphism will be deferred until more direct measurements of autolysis in N and O cells of *S. sanguis* are made.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant DE-02623 from the National Institute of Dental Re-

search and by a Public Health Service Career Development Award DE-70160 from the National Institute of Dental Research.

The author thanks Gary H. Cohen and Lewis Pizer for their generous help in the preparation of this manuscript. The excellent technical assistance of Mary Elchisak and Kristine Lillmars is also acknowledged.

LITERATURE CITED

1. Bollum, F. J. 1959. Thermal conversion of non-primary DNA to primer. J. Biol. Chem. **234**:2733-2734.
2. Burton, K. 1968. Determination of DNA concentration with diphenylamine, p. 163-166. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 12, part B. Academic Press Inc., New York.
3. Carlsson, J. 1968. A numerical taxonomic study of human oral streptococci. Odont. Revy **19**:137-160.
4. Cohen, S. S., and H. D. Barner. 1954. Unbalanced growth in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **40**:885-893.
5. DeStoppelaar, J. D. (ed.). 1971. *Streptococcus mutans, Streptococcus sanguis* and dental caries, p. 13-24. *In* Preventive tandheelkunde. Rijksuniversiteit. Utrecht, Netherlands.
6. Ekstedt, R. D., and G. H. Stollerman. 1960. Factors affecting the chain length of group A streptococci. I. Demonstration of a metabolically active chain splitting system. J. Exp. Med. **112**:671-686.
7. Fan, D. P. 1970. Autolysin(s) of *Bacillus subtilis* as dechaining enzyme. J. Bacteriol. **103**:494-499.
8. Hirota, Y., A. Ryter, and F. Jacob. 1968. Thermosensitive mutants of *E. coli* affected in the processes of DNA synthesis and cellular division. Cold Spring Harbor Symp. Quant. Biol. **33**:677-693.
9. Inouye, M. 1972. Reversal by sodium chloride of envelope protein changes related to DNA replication and cell division of *Escherichia coli*. J. Mol. Biol. **63**:597-600.
10. Lominski, I., J. Cameron, and G. Wylie. 1958. Chaining and unchaining of *Streptococcus faecalis* - a hypothesis of the mechanism of bacterial cell separation. Nature (London) **181**:1477.
11. Mendelson, N. H., and R. M. Cole. 1972. Genetic regulation of cell division initiation in *Bacillus subtilis*. J. Bacteriol. **112**:994-1003.
12. Mitchison, J. M. 1961. The growth of single cells. III. *Streptococcus faecalis*. Exp. Cell Res. **22**:208-225.
13. Nagoi, K., and G. Tamura. 1972. Mutant of *Escherichia coli* with a thermosensitive protein in the process of cell division. J. Bacteriol. **112**:959-966.
14. Pooley, H. M., and G. D. Shockman. 1969. Relationship between the latent form and the active form of the autolytic enzyme of *Streptococcus faecalis*. J. Bacteriol. **100**:617-624.
15. Reeve, J. N., D. J. Groves, and D. J. Clark. 1970. Regulation of cell division in *Escherichia coli*: characterization of temperature-sensitive division mutants. J. Bacteriol. **104**:1052-1054.
16. Rogers, H. J. 1970. Bacterial growth and the cell envelope. Bacteriol. Rev. **34**:194-214.
17. Rogers, H. J., M. McConnell, and I. D. J. Burdet. 1970. The isolation and characterization of mutants of *Bacillus subtilis* and *Bacillus licheniformis* with disturbed morphology and cell division. J. Gen. Microbiol. **61**:155-171.
18. Shaw, M. 1968. Formation of filaments and synthesis of macromolecules at temperatures below the minimum for growth of *Escherichia coli*. J. Bacteriol. **95**:221-230.
19. Smith, H. S., and A. B. Pardee. 1970. Accumulation of a protein required for division during the cell cycle of *Escherichia coli*. J. Bacteriol. **101**:901-909.
20. Tomasz, A. 1968. Biological consequences of the replacement of choline by ethanolamine in the cell wall of pneumococcus; chain formation, loss of transformability and loss of autolysis. Proc. Nat. Acad. Sci. U.S.A. **59**:86-93.
21. Van Alstyne, D., and M. I. Simon. 1971. Division mutants of *Bacillus subtilis*. Isolation and PBSI transduction of division-specific markers. J. Bacteriol. **108**:1366-1379.