DEVELOPMENT OF DEFINED MEDIA FOR MOTILITY AND FOR GROWTH OF *SPIRILLUM VOLUTANS*, WITH SPECIAL REFERENCE TO BIOLOGICAL MONITORING OF POLLUTANTS AND TO OBLIGATE MICROAEROPHILISM OF BACTERIA

by

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INTRODUCTION

The two main phases of my study were the development of an improved defined motility medium and the construction of a defined growth medium for Spirillum volutans. For precise and reproducible physiological studies, defined media have an advantage over media containing peptone or other substances derived from living organisms, because these substances may vary in precise chemical composition.

Caraway and Krieg (13) described a defined motility medium (DMM) which they used in studies of the phenomena of flagellar coordination and uncoordination in S. volutans. One main objective of my study was to alter this DMM to obtain increased sensitivity of cell motility and flagellar coordination to toxic materials. A second objective was the development of a standardized method employing the altered DMM for biological monitoring of pollutants. Such a method would be the first practical application of studies of bacterial flagella and could provide a rapid, simple method for detecting spills of toxic materials in industrial effluent.

Spirillum volutans has long been known to have unusual nutritional requirements. The cultivation of this organism in pure culture, free from association with other bacteria, was a great achievement, accomplished by Wells and Krieg (68) through recognition of its obligate microaerophilism. A main objective of my study was to develop a reliable defined growth medium which could be used in future nutritional and physiological studies. Wells (M. S. Thesis, Virginia Polytechnic Institute and State University, 1966) obtained growth of S. volutans in a defined medium containing a large number of amino acids and vitamins,

but these results could not be repeated. The probable explanation for this inconsistency became evident in the present study with the elucidation of the effect of autoclaved tyrosine.

A reliable defined medium was needed for further studies of the nature of the obligate microaerophilism of S. volutans. Obligately microaerophilic bacteria, defined as those which possess a strictly oxidative energy-yielding metabolism and yet are unable to grow in an aerobic or anaerobic atmosphere, but require lowered oxygen tension, are not common. Perhaps the best known are S. volutans and Campylobacter fetus. These bacteria must be distinguished from those (lactobacilli, streptococci, etc.) which obtain energy by fermentative processes and may grow better at lowered oxygen tension than in an air atmosphere; such organisms may better be referred to as aerotolerant anaerobes.

Another unusual characteristic of S. volutans is the extreme toxicity of orthophosphate to the organism's growth and respiration. A possible relationship between these two unusual characteristics was suggested by Hylemon et al. (38). The development of a defined medium, in which known levels of phosphate could be incorporated to determine the effect on oxygen tolerance, was therefore a useful objective, not only for the additional information which could be gained concerning the nutrition of S. volutans, but also because of the possibility that such data might be applicable to the phenomenon of microaerophilism and to oxygen toxicity in general.

REVIEW OF THE LITERATURE

Isolation and Cultivation of Spirillum volutans

Ehrenberg (21) in 1838 gave a description of Spirillum volutans by which the organism can still be recognized today. However, attempts to isolate this large and conspicuous bacterium were not successful until 1962, when Rittenberg and Rittenberg (55) took advantage of its rapid motility and aerotaxis in an ingenious capillary tube isolation procedure. They isolated the spirillum from a mixed culture which they had maintained in Pringsheim's soil medium for more than ten years, and which had been derived by Pringsheim from the cooling tower water of a sugar beet refinery in England. The isolated spirilla failed to grow in any of a large number of media tested, but they grew well when suspended inside a dialysis sac in mixed or pure cultures of E. coli or other bacteria. Exhaustive efforts failed to discover any nutrient or medium which could substitute for the presence of the other bacteria. Cell-free extracts of E. coli could substitute for the presence of a growing E. coli culture.

Rittenberg and Rittenberg noted that S. volutans did not form colonies on solid media, even in the presence of the mixed flora of the Pringsheim's medium culture. In two-membered cultures with E. coli, S. volutans grew well if the cultures were contained in test tubes, but grew poorly if 25 ml of medium was placed in a 250-ml Erlenmeyer flask, and not at all if the flask was shaken. Inhibition of growth of S. volutans in the mixed culture by 0.01 M phosphate was noted also.

Cultivation of S. volutans in an environment free of other bacteria was accomplished by Wells and Krieg (68), who were the first to recognize

the obligately microaerophilic nature of the organism. Using the capillary tube procedure, they isolated a strain from pond water hay infusion and cultured it in nutrient broth in sidearm flasks in which the atmosphere had been adjusted to contain 3% or 6% oxygen; the portion of air removed from the flask was replaced by nitrogen. The organism was found to grow under atmospheres containing from 1% to 9% oxygen, but it did not grow anaerobically or aerobically.

Wells (M. S. thesis, Virginia Polytechnic Institute and State University, 1966) supplemented peptone with a variety of carbon sources and found that only succinic and fumaric acids stimulated growth, while only citric acid was decidedly inhibitory. He devised a peptone-succinate-salts (PSS) medium of the following composition (g/l): peptone, 10.0; succinic acid, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; $MnSO_4 \cdot H_2O$, 0.002; $FeCl_3 \cdot 6H_2O$, 0.002. The pH was adjusted with KOH to 6.8. NaOH was not used because spirilla have been reported (57, 19) to be sensitive to sodium ions at low concentrations. This medium was used in subsequent studies of the growth (46) and flagellar activity (42) of the organism.

Wells also reported (M. S. thesis, Virginia Polytechnic Institute and State University, 1966) that "vitamin-free" acid-hydrolyzed casein at a concentration of 0.025% supported growth of S. volutans when substituted for peptone in PSS medium, but that higher concentrations (0.05%) of casein hydrolysate were inhibitory.

Caraway and Krieg (13) described a modified PSS (MPSS) medium, containing peptone at 5 g/l, which produced higher cell densities of S. volutans.

A comprehensive description of the cultural and physiological characteristics of the organism was geven by Hylemon et al., who proposed (37) that S. volutans be designated the type species of the genus Spirillum and that the Wells strain (ATCC 19554) be designated the neotype strain of S. volutans. The organism has been so designated by the Judicial Commission (40).

Motility and Flagellar Coordination of Spirillum volutans

Metzner (48) in 1919 described the reversing motility of S. volutans. The flagellar fascicles at each pole form oriented cones of revolution which reverse orientation simultaneously when the organism reverses direction, so that the cell moves smoothly back and forth and always possesses a "head"-oriented and a "tail"-oriented fascicle of flagella. Metzner also studied the effects on motility of certain compounds which were capable of uncoordinating the flagella, producing two "head" or two "tail" fascicles which opposed each other in propulsion so that the organism was incapable of translational motion, although the flagella continued to revolve rapidly. Krieg, Tomelty, and Wells (42) extended the list of uncoordinating agents, quantified their optimal effective levels, and reported that an agent that produced dual-head uncoordination could act on the cell in combination with a dual-tail agent to produce a null point at which coordination of the flagella again was possible. They also documented the simultaneity of the reversal of orientation of the flagella at both poles by cinephotomicrographic evidence.

Caraway and Krieg (13) studied the phenomenon of uncoordination

more extensively, developing a non-nutrient defined motility medium (DMM) which supported motility for at least 12 h and had the following composition (g/l): $MgSO_4 \cdot 7H_2O$, 1.0; $(NH_4)_2SO_4$, 1.0; $MnSO_4 \cdot H_2O$, 0.002; $FeCl_3 \cdot 6H_2O$, 0.002; ethylenediaminetetraacetic acid (EDTA), 0.029; and N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) buffer, 2.13; pH adjusted to 6.8 with KOH.

Biological Monitors for Industrial Pollutants

An early approach to assessment of water quality by means of the responses of organisms was the "saprobian systems" concept of Kolkwitz and Marsson (1908; reviewed by Patrick, 52). These investigators enumerated various zones of degradation in rivers recovering from organic pollution and listed algal species which were said to be characteristic of these various zones. Because of the large number of factors operating in aquatic communities, however, individual species can rarely be said to occur solely under a particular set of "polluted" or "non-polluted" conditions. The trend among later workers has been to study the structure of entire communities. Patrick, a pioneer in this field, advanced in 1949 the principle that "healthy" streams contain large numbers of algal species, mainly diatoms, each represented by relatively few individuals. Polluted water, on the other hand, is likely to contain large numbers of individuals representing only a few species. She has recently reviewed the history and a mathematical model of this concept, as well as other methods for utilizing community structure among algae as an index of water quality (52). The use of various indices of species diversity among invertebrates for in-stream detection of

pollution has been reviewed by Gaufin (24).

Cairns and Dickson (9) described a rapid method (the Sequential Comparison Index) which can be used by persons untrained in taxonomy to assess the species diversity of bottom fauna. A method which lends itself to automation, using laser holography to evaluate diatom community structure, has recently been proposed by Cairns et al. and has been reviewed along with other techniques (10).

All of the systems mentioned thus far represent approaches to in-stream monitoring of water quality; these techniques could yield information about effects which have already taken place as a result of the discharge of a polluting effluent. Recently, much attention has been given to the desirability of monitoring effluent quality within the industrial plant prior to discharge. Jackson and Brungs (39) suggested the maintenance of fish (or other test organisms of local importance, such as oysters or shrimp) in aquaria receiving various dilutions of effluent in a continuous-flow system. They stated that such a system, using death or signs of distress in test organisms as a warning signal, would verify the biological acceptability of the effluent and also show possible synergistic effects of the mixture of effluent and receiving stream water, if upstream water were used as the diluent.

The importance of biological monitoring as a supplement to (but not a substitute for) monitoring of chemical and physical parameters has been discussed by Cairns et al. (11). These authors developed an in-plant monitoring unit using changes in the movement patterns and breathing rate of fish in aquaria as indicators of sublethal toxicity. The system

has been tested most extensively with zinc as the toxicant, and responses to a few parts per million of this metal were detected with a latency on the order of hours. Cairns, Lanza, Sparks, and Waller (12) have emphasized the desirability of developing monitors in which biological responses can be detected with greater rapidity.

Most uses of bacteria for the assessment of pollution in the past have involved coliform studies for the detection of fecal contamination; this and some more recent uses of bacteria as indicator organisms for other types of pollution within the stream have been reviewed by Bott (5). Where the responses of bacteria and other microorganisms to pollutants have been studied within the laboratory, usually relatively long-term growth effects have been studied. For example, Guthrie (30) studied the bacterial population balances existing in a reservoir and the stability of these balances under laboratory conditions in the presence of various pollutants. The Microbiological Inhibition Testing Procedure described by Marks (44) measures the effects of toxic components of wastewater on the ability of a bacterial inoculum to oxidize organic matter in a BOD bottle. The test uses polarographic measurements of dissolved oxygen in the bottle over a period of hours or days to predict the probable effect of a given water on the bacterial population in a water treatment plant. Patrick (52) has reviewed the use of algal bioassay systems, most of which measure effects of chemical and physical changes on reproductive or photosynthetic rates.

Oxygen Toxicity

Cellular mechanisms of oxygen toxicity in organisms of all types were reviewed in 1968 by Haugaard (34). He emphasized the universality of oxygen toxicity in some degree among all organisms and pointed out that evolution of the ability to live in an atmosphere containing oxygen involved the development of protective mechanisms against oxygen as well as of enzyme systems for utilizing it in metabolism. He discussed the rise and fall of interest in the mechanisms of oxygen toxicity with the occurrence of situations in which this toxicity has practical importance for man, such as in diving and submarine escape operations during World War II, or the more recent medical interest in retrolental fibroplasia in infants and in hyperbaric oxygen therapy, for example in infections with clostridia. The following paragraphs will be devoted to a summary of the main points of Haugaard's review.

Oxygen at pressures above 1 atmosphere inhibits respiration in slices of a variety of tissues. Even before this effect becomes apparent, and as early as 15 min, the activity of glyceraldehyde-3-phosphate dehydrogenase may be inhibited so as to cause significant changes in the concentrations of glycolytic intermediates, lactate, and ATP. Tissue cells not directly exposed to the atmosphere or to arterial blood normally function at a partial pressure of oxygen less than that of the surrounding atmosphere; consequently, toxic effects can be demonstrated in cells of some tissues at oxygen pressures only slightly higher than that found in air. Tissue cultures of L-strain fibroblasts under an atmosphere containing more than 5% oxygen show a delay in the

onset of growth, and this lag increases as the amount of oxygen is increased.

Most aerobic bacteria are much more resistant to oxygen than mammalian cells; for example, exposure of staphylococci to 2 atm O_2 had to be extended for 6-8 h for inhibition of growth to occur.

Oxidation of sulfhydryl groups to form disulfides is widely regarded as a mechanism important in the toxicity of oxygen to cells. Essential sulfhydryl groups are found in coenzymes such as Coenzyme A and lipoic acid and in many enzymes, including urease, papain, arginase, and the flavoprotein dehydrogenases (for example, succinic dehydrogenase). Sulfhydryl oxidation is catalyzed by certain metal ions, including ferrous, ferric, and cupric ions; other ions, such as Co^{++} and Mn^{++}, do not have this effect and have been reported to protect against oxygen toxicity in a variety of animal systems, both *in vivo* and *in vitro*. Pyridoxal-requiring enzymes and ferredoxin also contain essential sulfhydryl groups and are inactivated by oxygen. The chemical mechanisms of sulfhydryl oxidation were discussed by Haugaard in some detail.

Lipid peroxidation is another mechanism which causes damage to cells, mainly membrane effects which result in permeability changes, swelling of mitochondria, and damage to proteins. It is thought that during the metal-catalyzed oxidation of compounds such as ascorbic acid or sulfhydryl compounds, lipid peroxide free radicals of the type $RO_2\cdot$ are formed. This process is greatly facilitated by ferrous iron.

Much emphasis has been placed on the role of reactive free radicals in destruction by oxygen of essential cellular components. In this

connection, it was pointed out that oxygen, while having the highest oxidation-reduction potential of all oxidants, is at the same time one of the most sluggish because of the strong bond between its atoms. Free radicals formed from the oxidizable substrate (as by light, heavy metals, or ionizing radiation) are necessary to initiate oxidation by molecular oxygen.

The role of the superoxide radical in oxygen toxicity has been reviewed recently by Fridovich (23). This radical, $O_2^-\cdot$, is produced by the univalent reduction of molecular oxygen and is formed as an intermediate in a number of biological oxidations, such as flavoprotein dehydrogenase reactions. The superoxide radical has been implicated in various deleterious effects on the cell, such as oxidation of thiols, destruction of DNA, peroxidation of lipids, and production of hemolysis in rat erythrocytes. McCord, Keele, and Fridovich (45) have presented data to support the theory that all oxygen-metabolizing organisms possess the enzyme superoxide dismutase as a biological defense mechanism against this radical. Among the microorganisms assayed by these investigators, all of the aerobes contained both catalase and superoxide dismutase activity. These activities were absent in the strict anaerobes studied. Aerotolerant anaerobes (Butyribacterium, Zymobacterium, Lactobacillus, and five species of Streptococcus) all lacked catalase, but all except L. plantarum possessed superoxide dismutase, although the levels were lower than in the aerobic (and facultative) organisms studied. The absence of superoxide dismutase and of catalase in L. plantarum was explained by the observation that this organism exhibited

no oxygen consumption; it was postulated that an organism incapable of reducing molecular oxygen would not need enzymes protective against the products of such reduction.

In some organisms, superoxide dismutase has been shown to be inducible by oxygen and to provide increased protection against oxygen toxicity when induced. Rats, ordinarily killed by exposure to an atmosphere of 100% O_2, survived this treatment if first kept for several days in an atmosphere containing 85% O_2, and assays of their lung tissue showed a parallel increase in the level of superoxide dismutase (23). Gregory and Fridovich (27, 28) showed that superoxide dismutase is inducible by oxygen in Streptococcus faecalis, which contains no catalase, and in E. coli, in which catalase is not inducible by oxygen. In Bacillus subtilis, however, catalase is inducible by oxygen, while superoxide dismutase is present but does not increase in response to increased oxygen tension. As evidence for an important role for superoxide dismutase in protecting cells against oxygen toxicity, the authors demonstrated that the increased levels of this enzyme allowed increased survival of induced E. coli and S. faecalis when subjected to hyperbaric oxygen. In contrast, the increased level of catalase in B. subtilis, in the absence of an increase in superoxide dismutase, was not protective.

E. coli contains two superoxide dismutases which are distinguishable by electrophoresis (3). Gregory, Yost, and Fridovich (29) showed that one of these enzymes contains manganese and is located in the matrix of the cell; it is inducible by increased oxygen tension. The other superoxide dismutase contains iron instead of manganese and is located in the

periplasmic space (released from the cell by osmotic shock). The level of this enzyme does not increase in response to oxygen. The levels of these two superoxide dismutases were subject to manipulation by varying the cultural conditions. Cells grown in an iron-deficient, aerated medium had high levels of the inducible mangano-enzyme but were deficient in the iron-containing periplasmic superoxide dismutase. In cells grown anaerobically in an iron-rich medium, the relative abundance of the two superoxide dismutases was reversed. It was shown that high levels of the iron-containing, periplasmic enzyme protected cells against superoxide radicals generated exogenously in the medium. In these experiments, exogenous superoxide was either produced by spontaneous re-oxidation of photochemically reduced riboflavin or generated enzymatically by xanthine oxidase. Cells low in iron-superoxide dismutase were killed by exogenous $O_2^-\cdot$ but could be protected by addition of bovine superoxide dismutase to the medium. The authors proposed that the physiological function of the constitutive iron-containing enzyme in the periplasmic space is to protect the cell against exogenous $O_2^-\cdot$, while the inducible mangano-enzyme of the cell matrix protects against superoxide generated by the cell in the course of its own metabolism.

Thomas, Neptune, and Sudduth (60) suggested that oxygen inhibition of respiration in animal tissues occurs by oxidation of the dithiol group of lipoic acid, thus inhibiting pyruvate and α-ketoglutarate dehydrogenases.

Chance (15) used a microspectrofluorometric method to record the fluorescence emitted from reduced nicotinamide adenine dinucleotide

(NADH) at 480 nm when illuminated with ultraviolet light at 366 nm. The degree of oxidation or reduction of nicotinamide nucleotides thus could be measured in situ in tissues exposed to anoxia or to hyperbaric oxygenation. Using this technique, Chance demonstrated an increased reduction of NAD in beating perfused rat heart when subjected to anoxia (perfused with N_2-saturated medium). A similar effect was produced by blocking the respiratory chain with Amytal, and in both cases the time course was very rapid; reduction was half-maximal within a few seconds. Return of the anaerobic heart to oxygenated conditions resulted in reoxidation of the NADH to the initial level within 4 min.

Hyperbaric oxygenation of tissues produced the opposite result. In liver, kidney, and brain of the rat, exposure to 9 atm O_2 for 5 min produced a shift toward oxidation of pyridine nucleotides. On depressurization over a 5-min interval, the NAD was reduced to the initial steady state.

Biochemical analysis of rapid-frozen biopsy specimens confirmed the fluorometric evidence and indicated that 65% of the total NADH + NADPH present under normoxic conditions was oxidized during hyperbaric oxygenation. Assay of glycolytic intermediates revealed no comparable changes in level and indicated that inhibition of glyceraldehyde-3-phosphate dehydrogenase is not involved in changes occurring during the short time span of the experiments.

The ratio of ATP to ADP + AMP was shown to rise dramatically under hyperbaric oxygen.

Chance explained these findings by postulating inhibition by oxygen

of an energy-requiring pathway of "reversed electron transport" first described by Chance and Hollunger (16). This pathway involves flow of electrons from succinate via Coenzyme Q and flavoprotein to reduce NAD. The energy for this "uphill" flow comes from a high-energy intermediate in electron transport which otherwise would phosphorylate ADP. Alternatively, the energy can be contributed by ATP itself by a reversal of phosphorylation.

According to Chance (15), it is the inhibition by oxygen of this pathway of reversed electron transport that is responsible for both the increase in oxidized pyridine nucleotide (the pathway is no longer operating to reduce NAD) and the increase in ATP level (the pathway is no longer consuming ATP). Furthermore, the inhibition of this pathway is proposed by Chance to be the mechanism of the immediate and short-term symptoms of oxygen poisoning in the intact animal (i.e., convulsions).

The functioning and inhibition of the reversed electron transfer pathway were demonstrated fluorometrically in a suspension of pigeon-heart mitochondria. Supplementation of the mitochondrial suspension with succinate and ATP produced an immediate increase in reduced NAD as measured by the fluorometer. A 40-second exposure of the mitochondria to 12 atm O_2, immediately before adding the succinate and ATP, inhibited the NAD reduction to less than 20% of that observed in the control experiment without hyperbaric oxygen.

A shift toward oxidation of pyridine nucleotides under hyperbaric oxygen might also be explained by the well-known inhibition of dehydrogenases by oxygen. Chance has shown, however, that inhibition of

succinate or ∝-ketoglutarate dehydrogenase, as measured by changes in the oxidation steady-state of cytochrome c, begins much more slowly than NADH oxidation and requires more than 45 min to reach a maximum. NAD is maximally oxidized within 5 min, a time course coinciding with the onset of convulsions in the rat.

A review of the biochemical aspects of oxygen toxicity, primarily in mammalian systems, was provided by Meijne (47) as a chapter in his book discussing the clinical applications of hyperbaric oxygen.

Gottlieb (26) has reviewed the effects of hyperbaric oxygenation on microorganisms, also from a clinical perspective. He presented a brief summary of studies of various mechanisms of oxygen toxicity in bacteria (sulfhydryl oxidation, respiratory inhibition, peroxide and free-radical formation, membrane effects, etc.). He also tabulated a number of studies which reported the effects of hyperbaric oxygen on a variety of microorganisms under various experimental conditions. He cited several studies which indicated that hyperbaric oxygen lengthens the lag phase of growth in a variety of bacteria and that a rise in E_h has the same effect on Clostridium perfringens; logarithmic growth, once initiated, was similar in all of these studies to control cultures not subjected to high oxygen concentration or E_h.

O'Brien and Morris (51), in studies with Clostridium acetobutylicum, showed that oxygen sensitivity in this anaerobe was in fact a response to the presence of oxygen, and not to an increase in the oxidation-reduction potential of the medium. Using ferricyanide as an artificial electron acceptor, they were able to raise the E_h of an anaerobic culture

from -400 to +370 mV. Growth, glucose consumption, and product formation were unaffected by this treatment unless oxygen was introduced, in which case growth stopped immediately. Conversely, in the presence of 40 μM dissolved O_2 (a concentration inhibitory to growth), poising the E_h at -50 mV with dithiothreitol did not permit growth.

Harrison (33) has reviewed several studies in which the technique of continuous culture was used to investigate the responses of various microorganisms to oxygen tension in the medium. Hyperbaric oxygenation was not used in any of these studies, but the dissolved oxygen in the culture vessel was varied between anaerobiosis and air saturation by adjusting either the composition of the gas atmosphere or the stirring rate. It was found that for most of the organisms studied, varying the dissolved oxygen tension between about 20 and 100 mm Hg had little effect on cell metabolism (growth rate, Q_{O_2}, cell yield, extracellular products, etc.). However, in studies of E. coli, Klebsiella aerogenes, Candida utilis, and a strain of Pseudomonas, a "critical" dissolved oxygen level of about 2-10 mm Hg was found, below which the respiratory quotient rose substantially but the cell yield from substrate decreased. It was suggested that this phenomenon was an uncoupling of "either the ATP generation or utilization process," but no mechanism for these changes was proposed. A great decrease in the efficiency of energy metabolism also was noted at high growth rates brought about by high rates of dilution in continuous cultures of K. aerogenes. Harrison and Maitra (cited in 33) presented evidence that the ratio of ATP to ADP remains constant in K. aerogenes during increased respiration brought about by lowered oxygen tension.

A different response to oxygen tension was noted in the nitrogen-fixing species Azotobacter chroococcum and A. nidulans. These organisms showed an increase in Q_{O_2} at dissolved oxygen tensions greater than 15 mm Hg (pO_2 in atmosphere greater than 0.1 atm). It was suggested that these organisms increase their respiration rate in order to lower the oxidative potential inside the cell to protect the oxygen-sensitive nitrogenase from inactivation. Dalton and Postgate (reviewed in 33) proposed that there is a shift from a coupled cytochrome pathway to an uncoupled one at high partial pressures of oxygen.

In a study by Brown (7), cultures of E. coli were exposed to hyperoxia at levels ranging from 1 to 16 atm O_2, and oxygen consumption was measured polarographically. The respiration rates of cultures incubated in air or in an atmosphere of 95% O_2: 5% CO_2 were the same. Respiration of cultures exposed to 3 or 15 atm of O_2, plus 1 atm of 95% O_2: 5% CO_2, was rapidly inhibited, falling essentially to zero within 3 to 5 h, depending on the degree of hyperoxia. Cultures removed from hyperbaric oxygen rapidly recovered to a respiration rate as high as that of controls maintained in air throughout the experiment. The rate and degree of recovery of respiration rate was dependent on the duration of exposure to hyperoxia, but cultures exposed to 90 psi of O_2 for 20 min did not respire at all for 5 min after removal to an atmosphere of air; they then recovered within the second 5 min to a respiration rate which was 99% of the control rate. The author stated that the reversible nature of respiratory inhibition by hyperoxia supported his conclusion from a previous study that the oxidative phosphorylation machinery of E. coli

is not damaged by hyperoxia. However, cultures exposed longer to the hyperbaric oxygen environment required a longer time for recovery and recovered their respiration rate to a lesser extent, suggesting possible secondary damage, according to the author. Respiratory inhibition and recovery occurred both in the presence and in the absence of a nitrogen source. The author stated that "because of the rapid reversal of inhibition upon removal from hyperoxia, it is tempting to speculate that oxygen is interacting at the biochemical level through a simple 'mass action' principle."

In a previous study by Brown and Howitt (8), the absence of CO_2 was shown to be toxic to E. coli; cell death occurred not only in an atmosphere of pure oxygen, but also in pure nitrogen and in CO_2-free air. This death could be prevented by the addition of CO_2 to any of these atmospheres. Large inocula survived better than small under these conditions and could protect the smaller inocula when separated except for gaseous diffusion between the two culture vessels; a sodium hydroxide trap for CO_2 abolished this protection.

In a search for the mechanism of the oxygen sensitivity of Spirillum volutans, Cole and Rittenberg (19) compared its respiration with that of two aerobic spirilla, Aquaspirillum itersonii and A. serpens. They used a medium of the following composition (g/l): sodium succinate, 1.0; peptone, 10; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.002; and $MnSO_4 \cdot H_2O$, 0.002; pH adjusted to 6.8 with KOH. These authors reported a lower rate of oxygen consumption for S. volutans than for the other two spirilla (30 nmole/mg protein/min, measured polarographically in

50 mM phosphate buffer with succinate as substrate, compared to 127 for A. serpens and 60 for A. itersonii). They also reported that S. volutans had low levels of all tricarboxylic acid cycle enzymes assayed, relative to the other two spirilla; fumarase and malate dehydrogenase were particularly deficient. All three spirilla had cytochromes b and c, but not cytochrome a; the level of cytochrome c was relatively low in S. volutans. S. volutans also had relatively low levels of NADH oxidase, catalase, and cytochrome oxidase activity. The latter was still higher than the respiration rate, however, and the authors stated that cytochrome oxidase is unlikely to limit the rate at which S. volutans can reduce oxygen.

Cole and Rittenberg suggested that the slower growth and respiration rates of S. volutans could be explained on the basis of the low levels of fumarase, malate dehydrogenase, and NADH oxidase in this organism. With succinate as the major energy source, the above deficiencies leave succinic dehydrogenase as the primary supplier of electrons to the electron transport chain. The low absolute level of this enzyme, then, could result in a low yield of ATP and low growth and respiration rates. The low respiration rate, the authors suggested, would allow a higher intracellular concentration of oxygen, and thus a cellular component equally sensitive to oxygen in all three spirilla would be damaged in S. volutans at a lower external oxygen concentration than that required to inhibit the aerobic spirilla.

Cole (18) reported further work concerning the microaerophilism of Spirillum volutans. Using the medium employed previously by Cole and Rittenberg, he found that 2% (v/v) inocula grew in medium equilibrated

with air if the inoculum was taken from an early exponential phase culture which had been incubated for 12 h under microaerophilic conditions. A fivefold decrease in dissolved oxygen occurred during the first 8 h of growth. Inocula from 48 to 72-h microaerophilic cultures initiated growth only if the medium had been boiled to expel dissolved oxygen. The growth rate of cultures incubated under microaerophilic conditions for the first 12 h, however, was stimulated by subsequent aeration on a rotary shaker. This method of aerating exponential phase cultures was employed to study the effect of oxygen on cell yield, enzyme activities, and synthesis of DNA and protein.

Cultures incubated under microaerophilic conditions (medium previously boiled and cooled; incubated statically) for 12 h and then aerated for 8 or 12 h were compared, with respect to the parameters mentioned above, to cultures incubated under microaerophilic conditions for 18 h. Growth yield (mg dry weight/l) after 12 h of aeration was three times the yield obtained in the microaerophilic culture. Respiration after 8 h of aeration was twice the rate in the microaerophilic culture, on a volume basis, but by 12 h of aeration the respiration rate had fallen to half the microaerophilic level. Addition of succinate to the medium after 8 h of aeration increased the growth yield and respiratory rate, and the spent medium supported growth only if more succinate was added. These results suggested, according to the author, that succinate was exhausted after 8 to 12 h of aeration. Arginine and thymidine incorporation, per ml of culture, was increased after 12 h of aeration to a value more than double that in the microaerophilic culture. Aeration for 8 h increased the specific activities of fumarase, malate dehydro-

genase, aconitase, and isocitrate dehydrogenase; succinic dehydrogenase activity was decreased slightly. After 12 h of aeration, however, fumarase and malate dehydrogenase activities were undetectable, while the other enzyme activities had not changed appreciably from the 8-h level.

Cole proposed the following hypothesis to explain the microaerophilism of S. volutans. Under microaerophilic conditions, the oxidation of succinate reduces oxygen to water. When the supply of succinate is depleted, the rate of oxygen reduction decreases, allowing the accumulation of oxygen or toxic oxidation products within the cells. If this accumulation results in the destruction of key catabolic enzymes, such as fumarase and malate dehydrogenase, then ATP synthesis would be insufficient to maintain viability. Loss of viability would be prevented by transferring cells to fresh medium under microaerophilic conditions, but accelerated by increased exposure to oxygen, particularly in the absence of an oxidizable substrate.

Assimilation of Iron by Bacteria

This subject has been reviewed by Lankford (43). Although iron is the fourth most abundant element in the earth's crust, in the ferric state it is extremely insoluble at neutral and alkaline pH. Microorganisms are known to excrete two general classes of compounds which chelate ferric iron, forming complexes from which the metal can be assimilated by the cell. Hydroxamate derivatives are produced by a variety of fungi, by mycobacteria and actinomycetes, and by other bacteria; these compounds are known collectively as siderochromes. Phenolic acid iron chelators

have been studied most extensively in the Enterobacteriaceae but are produced by a variety of bacteria. These compounds are derivatives of 2, 3-dihydroxybenzoic acid, commonly conjugated to amino acids such as glycine, serine, or lysine. Perhaps the best known microbial product of this type is enterochelin (enterobactin), a cyclic triester of 2,3-dihydroxybenzoyl serine produced by E. coli, Enterobacter aerogenes, and Salmonella typhimurium. Anaerobic bacteria have not been found to produce chelators for ferric iron; presumably the iron in their environment is in the ferrous state.

Competition for the available iron is an important factor in the host-parasite interactions of infectious disease, as discussed by Weinberg (64, 66). Iron is sequestered in mammalian serum by chelators known as transferrins; the degree of saturation of the serum transferrin is decreased when the host is invaded by the mechanisms of absorbing less iron in the intestine and removing iron from the serum for storage in the liver (66). Chelation of iron by conalbumin in egg white is one means by which bacterial growth in eggs is resisted (2).

It is difficult to prepare media truly lacking in iron because of contaminating amounts present in other chemicals, particularly sugars and phosphates (43). However, the iron requirement for maximal growth of Gram-negative bacteria is generally given, as summarized by Lankford (43), as between 0.02 and 0.1 μg/ml (0.36 to 1.8 μM). The iron requirement of strongly aerobic organisms is somewhat higher, and aeration may increase the iron requirement of some facultative species.

Lankford (43) cited a number of physiological consequences of iron

limitation. Deficiency of iron in bacterial cultures results in severe decreases in the amounts of catalase and peroxidase, cytochromes, succinic dehydrogenase, and aconitase. Respiratory activity decreases, and the growth rate may change from an exponential to an arithmetic linear function. Cells of Clostridium perfringens, E. coli, Corynebacterium diphtheriae, and Mycobacterium smegmatis have been reported to become elongated and filamentous in iron-deficient cultures. In the latter organism this morphological change was related to curtailment of DNA synthesis, which was aggravated by high aeration rates.

Maximal production of iron chelators by bacteria generally occurs when the iron concentration in the medium is barely sufficient to support maximal growth. Requirements for iron chelating compounds have been demonstrated in auxotrophic mutants, and stimulation by these compounds is a characteristic of some organisms which are capable of producing their own chelators to a limited degree. The latter type of requirement usually manifests itself when small inocula are used in defined growth media, but the requirement may not be apparent in complex media.

Stimulation by iron chelators has been extensively studied by Lankford and coworkers using Bacillus megaterium. This organism, when transferred from a complex to a defined medium, exhibited a lag phase of 3 to 4 h which was not dependent on the size of the inoculum. In addition, an inoculum-dependent lag period was noted; it could be reduced in duration by using a large inoculum or by adding filtrate from a mature culture of the same organism grown in the defined medium. B. subtilis var. niger behaved similarly, except that small inocula were completely

unable to grow in the absence of culture filtrate. The filtrate factor, schizokinen, later shown to be a hydroxamate, was secreted at an exponential rate by inoculum cells during the lag phase until a critical concentration was reached and exponential growth commenced. At this time, schizokinen synthesis ceased for the first 4 to 8 cell divisions and then resumed and continued until the maximal stationary phase of growth was reached.

Very small inocula (less than 10^3 cells/ml) of B. megaterium grew erratically, exhibiting a lag phase much longer than would be expected from the rate of schizokinen synthesis per cell in lag-phase cultures. Often these small inocula did not grow at all. Lankford hypothesized that the necessity for producing schizokinen at an exponential rate may damage the cells in very small inocula by competing with other essential biosynthetic pathways for common intermediates. These cells also face the problem of diffusion of intermediates into the medium. Supplementation of the medium with mixtures of amino acids, and particularly with phenylalanine and tryptophane, permitted growth of smaller inocula and shortened both the inoculum-independent and the inoculum-dependent components of lag. Lankford compared these results to those of Eagle (20), who found that several lines of cultured mammalian cells exhibited a critical population density below which they failed to grow unless specific amino acids were added; these amino acids apparently were being lost by diffusion from small inocula at a rate faster than their synthesis.

Another organism in which the size of the inoculum plays a critical

role in the initiation of growth in the absence of an iron chelator is Francisella tularensis. Halmann, Benedict, and Mager (31) reported that the minimum inoculum size required to initiate growth in a cysteine blood medium varied among strains from 10^5 to 10^8 organisms/5 ml. Growth from inocula of about 100 organisms was obtained by adding 10% (v/v) of filtrate from a culture of the same organism grown for 48 h in the same medium. When the inoculum size was held constant, the length of the lag phase was inversely proportional to the amount of filtrate added, but growth rate and yield were constant.

The growth initiating substance (GIS) from the filtrate was characterized by Halmann and Mager (32) and found to form complexes with both ferric and ferrous iron. It did not appear to be a hydroxamate, but various siderochromes permitted the growth of inocula of intermediate size, as did added ferrous or ferric iron. Because larger amounts of GIS per cell were required for initiation of growth from smaller inocula, Lankford (43) suggested that perhaps diffusion of intermediates from the cells impaired synthesis of GIS progressively as the inoculum size was decreased. He pointed out that such an effect would not be surprising in cultures of an organism whose natural habitat is the enclosed space of phagocytic vacuoles.

Gorini and Lord (25) studied the nutrition of a Micrococcus species and noted that growth was greatly improved when the medium had been subjected to high temperatures in the autoclave. Tyrosine was found to be the medium component made stimulatory by this treatment. A number of ortho-dihydroxyphenyl compounds, added at concentrations ranging from

5×10^{-5} to 5×10^{-7} M, were able to replace autoclaved tyrosine. These compounds included catechol and its 4-fluoro, -chloro, -amino, and -cyano derivatives; dihydroxyacetophenone; dihydroxyphenylalanine (DOPA); protocatechuic acid; adrenalin (epinephrine); arterenol (norepinephrine); pyrogallol; and gallic acid. Dihydroxyphenyl compounds in which the hydroxyl groups are meta or para to each other were not effective, although resorcinol (1, 3-dihydroxybenzene), hydroquinone (1, 4-dihydroxybenzene), and gentisic acid (2, 5-dihydroxybenzoic acid) were not inhibitory when added at the above concentrations to medium containing catechol.

The authors noted that stimulation by o-dihydroxyphenyl compounds was an "all or nothing" phenomenon; if growth was initiated at all, no relationship existed between the initial concentration of such compounds and the final cell yield of the culture. The authors did not propose a mode of action for the dihydroxyphenyl compounds. They did not believe that the effect was related to iron assimilation because no growth occurred when 10^{-3} M Fe^{++} was substituted for the catechols. However, in the light of present knowledge concerning phenolic acid chelators for ferric iron, iron assimilation undoubtedly was the function of these compounds, as stated by Hutner (36) and by Lankford (43). Micrococcus lysodeikticus, probably closely related to the organism studied by Gorini and Lord (25), has a similar requirement (56, 43).

MATERIALS AND METHODS

Sources of Chemicals

Peptone, yeast extract, "Casamino Acids," bacteriological grade agar, and purified agar were purchased from Difco Laboratories, Detroit, Michigan.

"Vitamin-free, salt-free" acid-hydrolyzed casein in powder form was a product of Nutritional Biochemical Co. (NBC; now known as ICN Pharmaceuticals, Inc.), Cleveland, Ohio. Acid and enzymatic hydrolysates of "vitamin-free" casein were also purchased from the same source in 5% or 10% sterile aqueous solution. An acid-hydrolyzed "vitamin-free" casein in 10% solution was purchased from Calbiochem, Los Angeles, California.

DL-arterenol HCl (A grade), L-aspartic acid (A grade), bovine liver catalase (B grade), and oxalacetic acid (A grade) also were products of Calbiochem.

L-alanine, L-arginine HCl, L-asparagine, L-cystine, L-glutamic acid, glycine, L-histidine, L-leucine, L-lysine HCl, L-methionine, L-phenylalanine, L-proline, L-tryptophane, and thymine, all M. A. grade, were purchased from Mann Research Laboratories, Inc. (now the Schwartz-Mann division of the Beckton-Dickinson Company), Rockville, Maryland. The following chemicals were also purchased from Mann: p-aminobenzoic acid, NF grade; calcium pantothenate, no grade stated; niacin, USP; niacinamide, no grade stated; pyridoxine HCl, USP; riboflavin, USP; thiamine, USP; and vitamin B_{12}, USP.

L-aspartic acid, N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES buffer), biotin, L-cysteine, folic acid, L-glutamic acid, L-glutamine, hydroxy-L-proline, L-histidine, L-isoleucine, L-leucine, L-methio-

nine, L-phenylalanine, L-proline, pyridoxal HCl, L-serine, L-threonine, L-tryptophane, L-tyrosine, and L-valine were purchased from NBC. No grade of purity was stated.

L-alanine, L-glutamine, p-hydroxyphenylpyruvic acid, L-isoleucine, L- arterenol HCl, DL-arterenol HCl, L-β-3, 4 dihydroxyphenylalanine (DOPA), D-DOPA, DL-DOPA, L-epinephrine, DL-epinephrine, protocatechuic acid, and sodium bisulfite, all Sigma grade, were obtained from Sigma Chemical Co., St. Louis, Missouri.

The following chemicals, all reagent grade quality, were products of Fisher Chemical Co., Fair Lawn, New Jersey: acetic acid, ammonium acetate, boric acid, n-butanol, carbon tetrachloride, L-cysteine, diethyl ether, ethanol, ethylenediamine tetraacetic acid (EDTA), ferric chloride, formalin, hydrazine sulfate, hydroxylamine, isoamyl acetate, methylamine, 1-naphthol, 1-naphthylamine, Norit (activated charcoal), and sodium bicarbonate.

Obtained from the J. T. Baker Chemical Co., Phillipsburg, New Jersey, were the following (all reagent grade): ammonium sulfate, aniline sulfate, copper sulfate, ferrous sulfate, hydrochloric acid, lead acetate, magnesium sulfate, manganous sulfate, nickel chloride, potassium phosphate, sodium chloride, strontium chloride, uranyl nitrate, and zinc sulfate.

Bovine serum albumin was purchased from Pentex (Miles Laboratories, Inc.), Miles Research Division, Elkhart, Indiana.

Albimi Brucella Broth was obtained from Pfizer Laboratories, New York, N.Y.

Aniline oxalate was purchased from K & K Laboratories, Plainview,

New York. Nitrilotriacetic acid (NTA) and o-nitrophenol were obtained from Eastman Organic Chemicals, Rochester, N.Y. Dieldrin and DDT were products of the Shell Oil Co., Tulsa, Oklahoma. Phenol was purchased from Mallinckrodt Chemical Works, St. Louis, Missouri. "Aroclor 1242" was a gift of the Monsanto Co., St. Louis, Missouri.

Quality of Water

All water used in defined media, or for the final rinse of glassware to be used with defined media, was distilled water which had been passed through a Bantam standard multi-bed resin cartridge (Barnstead Co., Boston, Massachusetts). Water so treated is referred to in this dissertation as "deionized water."

Complex media were made with distilled water which had not been deionized.

Cleaning of Glassware

All glassware was washed in a Heinicke laboratory glassware washer with "7X" non-ionic detergent (Linbro Chemical Co., New Haven, Connecticut) and given a final rinse with distilled water. In addition, glassware used for preparation of stock solutions for nutritional or uncoordination studies was soaked for 24 h in concentrated HCl and then washed as described above, followed by a final rinse in deionized water. All tubes used for growing cultures in defined medium were cleaned by autoclaving for 15 min in a 1% solution of "7X" detergent, washed as described above with deionized water rinse, and subsequently not used again with peptone-containing media. Washing between uses was routine

with deionized water rinse.

All plastic screw caps and membrane filter holders were washed routinely with distilled water rinse and then boiled in three changes of deionized water and placed in paper towel-lined baskets for drying.

All glassware and caps were air dried in a glassware oven.

Complex Growth Media

Modified peptone-succinate-salts medium (MPSS) was that described by Caraway and Krieg (13).

Casein hydrolysate-succinate-salts medium (CHSS) was identical to MPSS except that 0.25% "vitamin-free, salt-free" casein hydrolysate (acid hydrolyzed, NBC Co.) was substituted for peptone.

Maintenance of Cultures

Cultures of Spirillum volutans were incubated at 30 C in a box incubator. When this study was begun, no method was available for preserving cultures of S. volutans except by continual transfer, as the organism did not survive freezing or lyophilization. Throughout my study, stock cultures of S. volutans were maintained by daily subculture of 1 ml into 75 ml of MPSS broth in 250-ml sidearm flasks from which the air was evacuated and replaced by a mixture of 6% O_2: 94% N_2 as described by Wells and Krieg (68). Additional stock cultures were maintained by weekly subculture in 20 X 125 mm screw capped Pyrex test tubes containing 10 ml of semisolid MPSS medium (0.15% agar).

Stock cultures for the bioindicator study were maintained in 80-ml quantities of CHSS medium contained in 250-ml cotton-stoppered Erlenmeyer flasks incubated statically in an air atmosphere. Inocula of 1.5

ml were transferred daily.

The Wells strain of S. volutans (ATCC 19554) was used for the present work. After development of the defined growth medium, the Pringsheim strain (ATCC 19553) was tested for growth in this medium. In late 1973, stock cultures of both strains were frozen in liquid N_2 by the new method of Pauley and Krieg (54).

Cultures of Campylobacter fetus strains obtained from Dr. R. M. Smibert were maintained in Brucella Broth (Albimi) made semisolid by addition of 0.15% agar. They were incubated at 37 C and transferred every 24 or 48 h, using a 1-loop inoculum.

Methods Used in Study of S. volutans as a Monitor for Pollutants

The defined test medium (DTM) and the standardized procedure developed for testing the effect of a chemical agent (or an industrial effluent) on motility of S. volutans were results of this study and will be described in that section.

Most of the potential pollutants tested were taken from a list of hazardous polluting substances prepared by the Environmental Protection Agency (61). Borosilicate glassware used with test compounds was cleaned with hydrochloric acid as described above. Slides were boiled in 1% HCl, thoroughly rinsed, and boiled twice in deionized water and dried. In the initial phases of the work, the concentrations of zinc ions used were verified by atomic absorption spectrophotometry.

Cells displaying normal motility and uncoordinated cells were photographed using the motility-track method of Vaituzis and Doetsch (62). Time exposures of 5 sec, Kodak Plus-X Pan film, and a 35-mm

Leica camera with microscope mount were used. The Leitz Ortholux microscope used was equipped with N.A. 1.20 darkfield condenser and 150-watt XBO xenon burner.

As a check on the reliability of the method, the usual practice was to select test solutions "blind" and at random, recording the results before noting the identity of the agent. In addition, two other tests of reliability were performed. In the first of these, twelve test solutions were prepared by a person other than the investigator. The coded solutions included uncoordinating agents at their minimum effective concentrations, uncoordinating compounds at levels too low to be effective, and also DTM blanks (no agents added). The number of solutions of each type also was unknown to the examiner. The second test was conducted in a similar manner, except that it was designed to show whether a person unaccustomed to working with these tests or with the test organism could correctly detect the presence of effective levels of pollutants. The person was instructed in the use of the microscope and was shown examples of normal and of uncoordinated preparations of S. volutans. He then was presented with eight test solutions to be examined using the standard method. These solutions included uncoordinating agents at their minimum effective concentrations, uncoordinating agents at sub-minimal effective concentrations, DTM blanks, and ineffective agents.

Methods Used in Studies of Nutrition and Microaerophilism in S. volutans

Stock solutions. Certain standard stock solutions were used repeatedly during the construction of the defined growth medium. As a

base for experimental defined media, a solution identical to MPSS with peptone omitted was used; this mixture was designated "succinate-salts."

The trace mineral solution of Hylemon et al. (38) also was used as a standard component of many experimental formulations of the defined medium. It will be designated in this dissertation as "trace minerals." The composition of the solution was as follows (expressed as final concentration in the medium, g/l): $CaCO_3$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.00072; $CuSO_4 \cdot 5H_2O$, 0.000125; $CoSO_4 \cdot 7H_2O$, 0.00014; H_3BO_3, 0.000031; and $Na_2MoO_4 \cdot 2H_2O$, 0.000245. This mixture was prepared as a 100X stock solution, dissolved by acidifying with HCl, and stored at 4 C until added to media.

The defined motility medium (DMM) of Caraway and Krieg (13) was used for washing cells whenever a washed inoculum was desired; it consisted of MPSS with the peptone and succinate omitted and with the addition of 10^{-2} M BES buffer (2.13 g/l) and 10^{-4} M EDTA (0.0292 g/l).

During the course of this study, many compounds were used experimentally in defined media. The concentrations of these components are expressed as per cent (w/v) unless otherwise noted.

A somewhat arbitrary stock solution of amino acids, hereinafter designated as the "eleven amino acid mixture," was used as a standard component of the defined medium during its developmental phases. This stock solution contained the L-enantiomers of the following amino acids, each at 0.2%: lysine, threonine, histidine, serine, isoleucine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, and methionine. The stock was prepared by first dissolving the isoleucine in a minimum

amount of 2 N HCl and then rinsing in each of the other amino acids with deionized water. Slight heating was required to achieve complete solution; the mixture was then cooled to room temperature before adjusting the pH to 6.8 with 2 N KOH and bringing to volume with deionized water. The stock solution was sterilized by filtration through a 0.45 μ membrane filter (Millipore Corp., Bedford, Mass.). The sterile solution was dispensed aseptically in 10-ml amounts into sterile screw-capped tubes and stored frozen. It was diluted ten-fold into experimental defined media, giving a final concentration of 0.02% of each amino acid; thus the combined concentration of all amino acids in the medium was 0.22%, approximating the 0.25% casein hydrolysate which the stock was designed to replace.

"Synthetic casein hydrolysate." Amino acids, vitamins, and other compounds to be tested as possible growth factors for S. volutans were prepared as concentrated stock solutions (100X for amino acids, 1,000 or 10,000X in the case of vitamins). Tubed aliquots of these solutions were stored frozen. For the construction of an experimental defined growth medium, stock solutions were combined at 2X the desired concentration and the pH was adjusted to 6.8 using 2 N KOH or 2 N HCl. The mixture was then sterilized by filtration and added to an equal volume of double-strength succinate-salts which had been sterilized by autoclaving.

Early attempts to construct a defined medium based on "synthetic casein hydrolysate" were carried out using 50 or 100-ml volumes of medium in sidearm flasks under an atmosphere of 6% O_2: 94% N_2. The

inoculum used was usually 0.5 ml of a 24-h culture grown in MPSS or in CHSS.

Stimulation studies. Beginning with the studies of growth stimulation by individual compounds, the use of large quantities of medium in flasks was discontinued. Media were instead prepared from filter-sterilized stock solutions by dispensing aseptically into 20 X 125 mm Pyrex screw-capped culture tubes. The succinate-salts base was prepared in double-strength concentration and sterilized by autoclaving at 121 C for 15 min; it was then dispensed in 2.5-ml quantities into sterile culture tubes. Various experimental medium components were then added from stock solutions, and sterile deionized water was used to bring the volume in each tube to 5.0 ml. In this way, large numbers of experimental defined medium formulations could be tested in duplicate simultaneously.

Tubes of defined media and controls containing casein hydrolysate were incubated in an air atmosphere at 30 C.

Preparation of inocula. The standard inoculum used for tubed media was one drop of a 100 Klett unit suspension of washed cells from a 24-h culture grown in MPSS under 6% O_2: 94% N_2. The cells were harvested by centrifugation at 750 X g for 5 min, washed twice in and resuspended in DMM to a turbidity of 100 Klett units as measured with a Klett-Sommerson colorimeter using a blue filter (420 nm) and 16-mm glass cuvettes. DMM was used as a blank. One drop of this washed cell suspension was inoculated into each tube using a sterile 1-ml serological pipette.

In order to relate the turbidity of inocula to actual cell numbers,

a hemacytometer (Hausser Improved Neubauer Counting Chamber) was used. After determining the turbidity in Klett units of serial dilutions of a washed cell suspension, the cells were fixed with one drop of formalin per 5 ml of suspension and counted in the chamber. Three separate aliquots of each suspension were counted over the entire grid area, and the mean cell number thus obtained was plotted against the turbidity to obtain Figure 1. Cell numbers given in connection with minimum inoculum size, etc., were derived from turbidity measurements using these data.

Estimation of growth. The presence or absence of growth in experimental formulations of defined media was determined by inspection and comparison with control media containing casein hydrolysate. Quantitative growth measurements for comparative purposes, growth curves, etc., were made turbidimetrically, using a Klett-Sommerson colorimeter as described above. In all cases, identical uninoculated media were used as blanks.

Cultures in which growth was observed were examined by darkfield microscopy to verify that they were pure cultures of S. volutans.

Gas chromatography. A 10% solution of "vitamin-free, salt-free" acid-hydrolyzed casein (NBC) was prepared by acidifying with H_2SO_4, extracted with ether, and subjected to gas chromatographic analysis for detection of volatile fatty acids and of alcohols. Methylation with absolute methanol followed by chloroform extraction was used to prepare volatile methyl esters of any non-volatile acids.

The instrument employed was a Hewlett-Packard Model 700 dual-column gas chromatograph (Hewlett-Packard/Analytical Instruments, Avondale, Pa.)

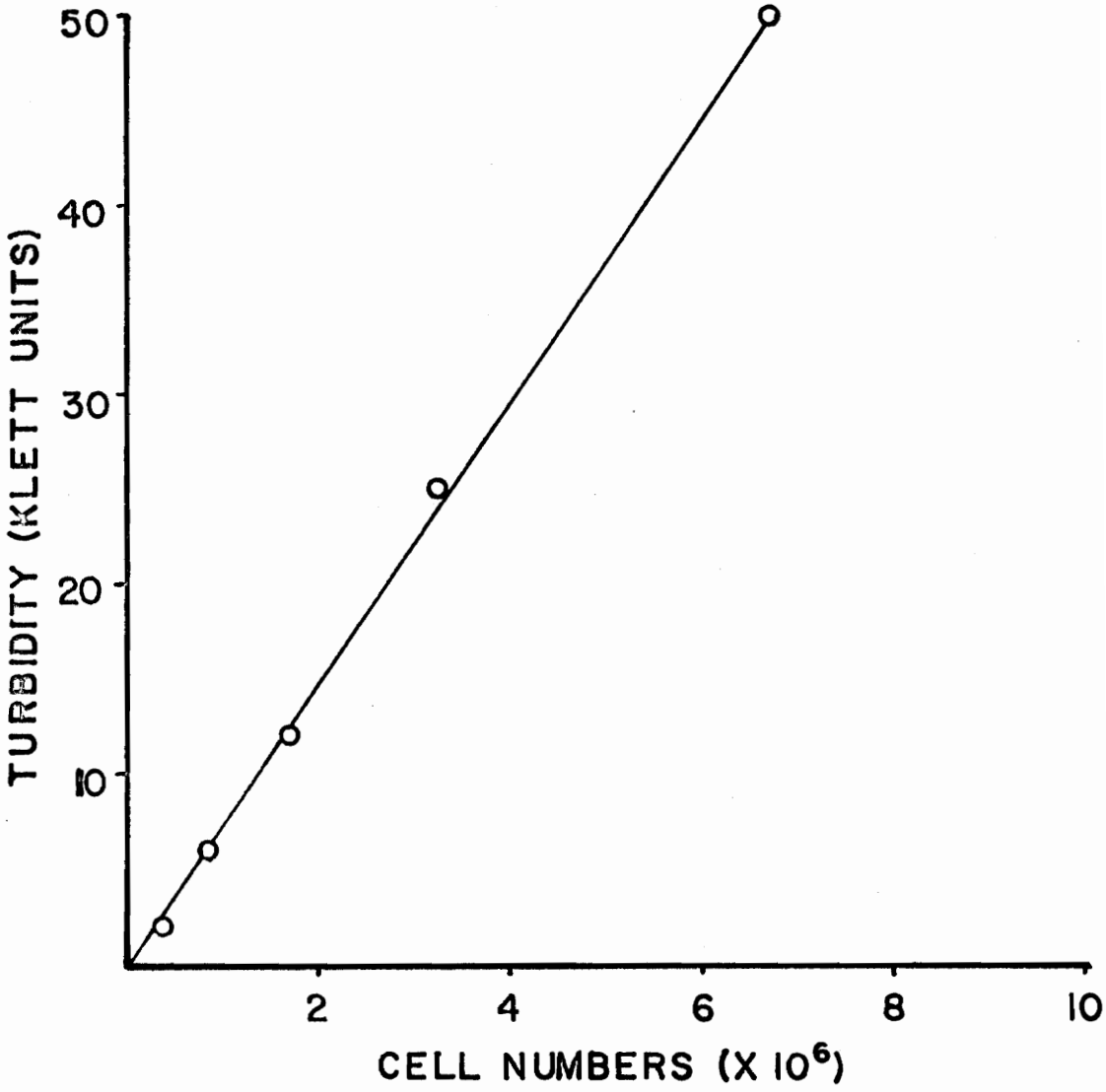


FIGURE I. RELATIONSHIP BETWEEN CELL NUMBERS AND TURBIDITY IN KLETT UNITS OF A WASHED SUSPENSION OF S. VOLUTANS. (CELLS CULTURED IN MPSS FOR 24 H UNDER 6% O_2; WASHED 2X IN AND RESUSPENDED IN DMM)

equipped with a flame-ionization detector. Samples (5 µl of extract) were injected into 6-ft, ¼-inch diameter glass columns packed with Resoflex LAC-1-R-296 (Burrell Corp., Pittsburgh, Pa.). Helium was used as the carrier gas with a flow rate of 10 cc/7 sec. The oven temperature was set for isothermal operation at 50 C for 4 min, then programmed to 130 C at a rate of 5 C/min using a Hewlett-Packard temperature programmer.

Paper chromatography. Tyrosine was dissolved in 0.5 ml of 2 N KOH, and the pH was adjusted to 9.3 with HCl before adjusting the final volume with deionized water to prepare a 0.3% solution. A portion of the tyrosine solution was autoclaved at 121 C for 15 min and compared with untreated tyrosine by paper chromatography.

Six µg of tyrosine (2 µl of solution) were subjected to descending chromatography for 14 h on Whatman No. 1 chromatography paper after overnight equilibration in a solvent system consisting of n-butanol: pyridine: water, 1:1:1 (v/v). (See Reference 69, vol. I, p. 297).

Nine and 18 µg of tyrosine were chromatographed in another solvent, consisting of n-butanol: ethanol: water, 5:1:1 (69, vol.I, p. 376) for 22.5 h.

A sulfanilic acid reagent (69, vol. II, reagent 64, p. 119) and a ferric chloride-ferricyanide reagent (69, vol. II, reagent 369, p. 160) were used as sprays for visual detection of spots on the paper chromatograms.

Assay of phosphate. Phosphate determination was carried out on 1% solutions of casein hydrolysate and peptone by the method of Fiske and

Subbarow (22) with the following modifications: (1) 10-ml volumes were used instead of 100 ml; and (2) one drop of Tween 80 was added to each tube (sample and standard). The Tween 80 was necessary to prevent the formation of a voluminous precipitate which otherwise appeared in solutions containing peptone. The blue color which appeared was measured as absorbance at 660 nm in a Model 139 Hitachi Perkin-Elmer spectrophotometer (Hitachi Ltd., Tokyo, Japan).

Polarographic measurement of dissolved oxygen. The concentration of dissolved O_2 in shaken flasks of defined growth medium was measured with a Model 125-A polarograph with a Model 17365 polarographic needle electrode (both obtained from Instrumentation Laboratories, Inc., Boston, Mass.). The electrode was calibrated for 0% O_2 using medium through which oxygen-free nitrogen was bubbled. Uninoculated medium which had been shaken in an air atmosphere with the cultures to be measured was used to set the polarograph to 100%, and the dissolved oxygen in culture flasks was measured relative to this control.

Methods Used in the Study of Campylobacter fetus

Iron and various compounds were added to Albimi Brucella Broth, with or without 1.5% agar, for determination of their effects on the growth of C. fetus. Broth medium was tubed in 5-ml amounts in 20 X 125 mm Pyrex culture tubes plugged loosely with cotton and inoculated with one loopful of growth removed from a stock culture in semisolid (0.15% agar) Albimi medium. Agar plates were streaked with a suspension of cells prepared by removing the upper layer of a 24-h culture in semisolid Albimi medium, which contained the organisms, and suspending the

cells evenly in 5 ml of Albimi broth by repeatedly drawing the suspension into a pipette. A loopful of this broth suspension was streaked onto each plate.

All cultures of C. fetus were incubated at 37 C. Broth cultures were incubated in a candle jar or in air. Streak plates were incubated in a candle jar or in sealed jars in which the air pressure was adjusted by means of a vacuum pump and a mercury manometer to provide varying amounts of oxygen with or without supplementary CO_2, as specified in the Results section.

In experiments performed for the purpose of comparing the effects of ferrous and of ferric iron, ferrous sulfate and ferric sulfate were sterilized by filtration and added aseptically to the medium after it had been autoclaved. In other experiments, ferrous sulfate was autoclaved in the medium, as were other compounds added experimentally.

RESULTS

Part 1. Development of a Defined Motility Medium and a Standardized Method for Use of Spirillum volutans as a Biological Monitor for Industrial Pollutants

Suspensions of S. volutans in the DMM of Caraway and Krieg (13) showed normal reversing motility for at least 12 h and were uncoordinated by zinc sulfate at a concentration of approximately 20 ppm. The first experiments in the present study were directed toward modification of this medium to allow uncoordination of the cells at lower concentrations of zinc while still maintaining normal reversing motility for extended periods of time in the absence of added toxicants.

When BES buffer was omitted from the medium, motility still appeared normal. The medium without BES was then diluted tenfold with deionized water. In this dilute medium, the cells showed unidirectional motility. A twofold dilution of DMM without BES permitted reversing motility, but the movement was jerky in comparison with the smoothly reversing motility of cells in DMM.

Omission of both BES and EDTA from DMM resulted in wobbly motility and less frequent reversal of direction than in DMM. The cells were found to display normal motility in a modification of DMM, lacking BES, in which magnesium sulfate was decreased to one tenth, and EDTA to one half, the concentration present in DMM. Reversing motility was obtained in this medium with the further omissions of ferric chloride and manganous sulfate.

Further decreases in concentration yielded a medium of the following

composition (g/l): $MgSO_4 \cdot 7H_2O$, 0.05; $(NH_4)_2SO_4$, 0.5; and EDTA, 0.0073. This medium maintained reversing motility for several hours and permitted uncoordination of most of the cells by 3 ppm of zinc.

The modified medium, however, was unbuffered. Extremes of pH (4.4 or 9.9) cause uncoordination of S. volutans in DMM (13). It was considered desirable to buffer the medium in order to observe the effects of pollutants on the cells independently from fluctuations in pH. Addition of 10^{-2} M BES (the concentration used in DMM), however, produced a medium in which a large percentage of cells exhibited wobbly reversing motility in the presence of 3 ppm of zinc (a concentration which uncoordinated cells in unbuffered medium). When 10^{-3} M BES was incorporated into the medium, more than 90% of the cells became uncoordinated in the presence of 3 ppm of zinc; those which did not appear uncoordinated showed slow, unidirectional motility. When 3 ppm of zinc was added (as $ZnSO_4 \cdot 7H_2O$) to unbuffered medium, the pH fell from 6.8 to 5.8; in the medium containing 10^{-3} M BES, the final pH was 6.7. BES at this concentration was, therefore, adopted as a standard component of the defined test medium (DTM).

NaCl at 10^{-2} M uncoordinated cells of S. volutans in DTM. An attempt was made to increase the sensitivity of the cells to zinc by incorporating 9×10^{-3} M NaCl, which did not affect motility by itself, into DTM. The minimum effective concentration of zinc, however, was 3 ppm both in the presence and in the absence of NaCl.

It was possible that dimethyl sulfoxide (DMSO) might facilitate entry of toxicants into the cells and increase the sensitivity of the

monitoring system. However, addition of 1% DMSO to DTM caused jerky reversing motility when used alone and did not increase the sensitivity of the cells to zinc.

DTM was adopted without further modification as the standard motility medium to be used throughout the remainder of the monitoring study. Its composition was as follows (g/l): $MgSO_4 \cdot 7H_2O$, 0.05; $(NH_4)_2SO_4$, 0.5; EDTA, 0.0073; and BES, 0.2133. The pH was adjusted to 6.8 with KOH. The medium was prepared at 10X concentration and was sterilized at 121 C for 15 min.

The standardized procedure developed for testing the effect of a chemical agent (or an industrial effluent) on motility was as follows. Cells from a 24-h CHSS culture were centrifuged at 750 X g in a clinical centrifuge, washed once in 80 ml of 1X DTM, and suspended in 80 ml of 1X DTM. The suspension was allowed to stand for 1 h in DTM before use. The same preparation of cells could then serve as a stock cell suspension for use throughout an 8-h working day; in fact, reproducible results have been obtained with cells that have remained in DTM for 10 h. The initial 1-h incubation was found to yield cells exhibiting more reproducible results.

Test compounds were prepared to 1.11X the desired test concentration in deionized water, and 9 parts of a test solution were then added to 1 part of 10X DTM. (In practice, a suitable dilution of industrial effluent could be used.) A 5-ml aliquot of stock cell suspension was centrifuged for 1 min, resuspended in the test solution, and immediately examined by darkfield microscopy at 125X. The minimum effective concen-

tration of a pollutant was defined as the minimum concentration necessary to eliminate reversing motility in more than 90% of the cells. This definition allowed for the presence of cells showing slow unidirectional motility, a condition which often preceded uncoordination of the flagella in low concentrations of agents and which was easily distinguishable from the normal rapid, smoothly reversing motility.

The minimum effective concentrations (MEC) of agents which were found to uncoordinate S. volutans are shown in Table 1. The uncoordinating effects of the metal pollutants were detected at 2 or 3 ppm. Certain other agents (cetyl pyridinium chloride, 1-naphthol) also were effective in this range, while others produced uncoordination at higher concentrations (ranging up to several per cent for the alcohols tested). All results were reproduced on numerous occasions.

Table 2 indicates levels of some potential pollutants which failed to uncoordinate the spirilla. The most notable of these were the long-range, bioaccumulated poisons Aroclor 1242 (registered trademark, Monsanto Co., St. Louis, Mo.; a polychlorinated biphenyl product) and the organochlorine insecticides Dieldrin and DDT. These compounds were insoluble in water and were dispersed in the aqueous medium after dissolution in 95% ethanol; the final test solutions consequently contained 0.475% ethanol in addition to the toxicant (10 ppm). These compounds appeared to form microscopic globules when dispersed in the medium and may thus have been prevented from contacting the cells effectively. Other compounds, such as phenol, which failed to uncoordinate spirilla even at concentrations as high as 90 ppm may possibly affect motility at higher concentrations (13).

TABLE 1

Minimum Effective Concentrations (MEC) of Uncoordinating Agents in DTM

Agent	MEC[1]	Comments
Hg^{++}	3 ppm	added as $HgCl_2$
Ni^{++}	3 ppm	added as $NiCl_2 \cdot 6H_2O$
Zn^{++}	3 ppm	added as $ZnSO_4 \cdot 7H_2O$
Cu^{++}	2 ppm	added as $CuSO_4 \cdot 5H_2O$
Pb^{+}	2 ppm	added as lead acetate
U^{6+}	1 ppm	added as $UO_2(NO_3)_2$
Cetyl pyridinium chloride	1 ppm	Delayed uncoordination (occurs within 5 min); flagellar activity ceases within 15 min
Sodium dodecyl sulfate (sodium lauryl sulfate)	200 ppm	Flagella completely immobilized and cells motionless; cells can swim at 100 ppm
"7X" detergent[2]	1%	Delayed uncoordination (occurs within 1 min)
1-Naphthol	3 ppm	
1-Naphthylamine	90 ppm	
Hydroxylamine	40 ppm	
p-Nitrophenol	25 ppm	
Hydrazine	10 ppm	added as hydrazine sulfate
Aniline	30 ppm	added as the sulfate or oxalate
n-Propanol	2%	Effects of alcohols progressed during a period of a few min from slow unidirectional swimming to dual-head uncoordination
Ethanol	4%	
Ethylene glycol	10%	

[1] ppm by weight; % by volume. Concentrations expressed as the ion or compound listed under "Agent."

[2] Linbro Chemical Co., New Haven, Connecticut.

TABLE 2

Agents Which Did Not Produce Uncoordination at Levels Tested in DTM

Agent	Concentration	Comments
Aroclor 1242[1]	10 ppm (in 0.475% ethanol)	no effect after standing for 2 h
Dieldrin	10 ppm (in 0.475% ethanol)	
DDT	10 ppm (in 0.475% ethanol)	
CCl_4	90 ppm	
isoamyl acetate	90 ppm	only effect was a slightly wobbly motility
nitrilotriacetic acid	90 ppm	neutralized with KOH; cells still normal after 75 min
H_3BO_3	90 ppm	
Sr^{++}	90 ppm	added as $SrCl_2 \cdot 6\ H_2O$
o-nitrophenol	90 ppm	yellow solution; visually detectable
methylamine	90 ppm	
phenol	90 ppm	

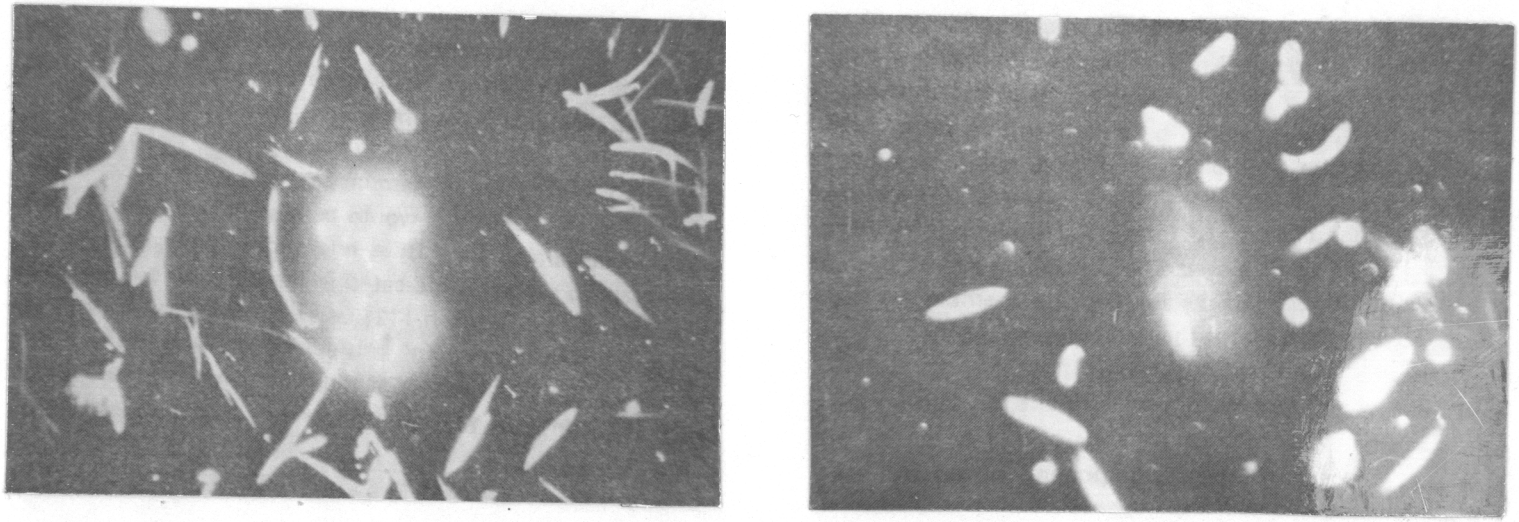
[1]Registered trademark, Monsanto Co., St. Louis, Missouri.

The reliability of the testing method was confirmed by the procedures described in Materials and Methods. In every case, correct characterization of the motility response in the coded samples was achieved.

It is unlikely that an industrial effluent would contain only one potential pollutant; therefore, the effects of combinations of agents on motility were studied. An additive effect was observed when any two of the metals were used together, each at ½ its minimum effective concentration (i.e., such mixtures produced uncoordination). Cetyl pyridinium chloride at 1 ppm, which normally caused only a delayed uncoordination within 5 min, uncoordinated the cells immediately in the presence of 1 ppm Zn^{++} (normally a sub-minimal effective concentration). A mixture of ethanol and n-propanol, each at ½ its minimum effective concentration, uncoordinated about half the cells; the rest were still able to swim but did not reverse direction.

In addition to additive effects, a synergistic effect was demonstrated between Zn^{++} and Ni^{++}, which produced a response in combination at levels well below the minimum effective concentrations of the individual agents. Uncoordination was caused by the simultaneous presence of 0.5 ppm Zn^{++} and 0.5 ppm Ni^{++}. Neither of these metals caused uncoordination alone at 1 ppm.

Preparation of a photographic demonstration of the lack of or presence of motility in testing for pollutants would be advantageous in that a permanent record would be available for future reference. The feasibility of preparing such a photographic record is indicated in Figure 2, where the time-exposure motility-track method of Vaituzis



A. B.

FIGURE 2. MOTILITY-TRACK PHOTOMICROGRAPHS OF S. VOLUTANS. MAGNIFICATION = 117 X. CELLS PREPARED FOR OBSERVATION BY THE STANDARD PROCEDURE DESCRIBED IN THE TEXT. A: DTM CONTROL. B: DTM + 3 PPM ZINC.

and Doetsch (62) was employed for normal cells and for cells uncoordinated by 3 ppm Zn^{++}. It can be seen that normal cells leave an exposure track on the film because of their motion, while uncoordinated cells do not swim and consequently appear as overexposed "blobs" on the film.

Part 2. The Nutrition of Spirillum volutans and its Application to the Study of Microaerophilism

Development of a Defined Growth Medium for Spirillum volutans

Substitution of casein hydrolysate for peptone in MPSS. As the first step toward development of a defined growth medium, it was desirable to substitute a simpler source of amino acids, such as casein hydrolysate, for the peptone in MPSS. When "vitamin-free, salt-free" acid-hydrolyzed casein purchased in powder form (NBC) was used at a concentration of 0.25% instead of peptone, growth comparable to that obtained in MPSS was obtained. The amount of growth was the same if 0.5% casein hydrolysate was used, but only barely visible growth occurred if the concentration of casein hydrolysate was lowered to 0.025%. Other acid and enzymatic hydrolysates of "vitamin-free" casein, purchased in the form of 5% or 10% sterile solutions and not designated as "salt-free," did not support growth of S. volutans when used at a final concentration of 0.25%. Casamino Acids (Difco) did not support growth when used at concentrations of 0.125%, 0.25%, or 0.5%.

The medium in which 0.25% "vitamin-free, salt-free" acid-hydrolyzed casein was substituted for peptone, and all other components were identical to those of MPSS, was designated "casein hydrolysate-succinate-salts" (CHSS) medium. In this medium, as in MPSS, densities of at least 8×10^6 cells/ml (50-60 Klett units) could be obtained in 24 h by serial transfer of 1 ml of the previous 24-h culture into 75 ml of medium under a 6% O_2: 94% N_2 atmosphere. Addition of 0.0125% yeast extract to CHSS as a source of vitamins and other possible growth factors

had no effect on the rate or amount of growth.

Growth in CHSS without special gas atmosphere. S. volutans grew under an air atmosphere much more readily in CHSS than in MPSS. In Erlenmeyer flasks containing 75 ml of medium and plugged with cotton or covered by loose plastic caps, cultures in CHSS could be maintained indefinitely by daily serial transfer of 0.5 ml. Cultures in MPSS under the same conditions grew sparsely and soon died out.

These observations were applied to the cultivation of S. volutans without a special gas atmosphere for the pollution monitoring studies described in Part 1 of this chapter. Stock cultures were, however, maintained by daily serial transfer of MPSS cultures under an atmosphere of 6% O_2: 94% N_2, and inocula for all defined medium studies were obtained from this source. In the initial attempts to devise a defined growth medium, experimental media were prepared in 75-ml or 100-ml quantities in sidearm flasks and were incubated under the 6% O_2 atmosphere in order to enhance the growth response if any occurred. Beginning with the supplementation studies (described below), experimental media and the defined growth medium eventually developed were tubed and incubated in an air atmosphere.

The effects of growth medium on oxygen tolerance will be described more fully in a subsequent section.

Attempts to substitute "synthetic casein hydrolysate" for acid-hydrolyzed casein in CHSS. Growth of S. volutans in succinate-salts with "vitamin-free, salt-free" casein hydrolysate suggested that a mixture of the individual amino acids found in casein might substitute for

the hydrolysate. Two amino acid mixtures were used: that listed by Wells (M. S. thesis, Virginia Polytechnic Institute and State University, 1966, p. 32; see Table 3a), and another list, given as the composition of casein by Bridson and Brecker (6; see Table 3b).

No growth occurred in media consisting of "synthetic casein hydrolysate" (either formulation) and succinate-salts, with or without the addition of 1.5×10^{-3} M K_2HPO_4. Neither CHSS nor MPSS contains any added phosphate; the organism's requirement presumably is met by contaminating phosphate in the other ingredients, particularly peptone or casein hydrolysate. When attempting to eliminate these complex ingredients, I added phosphate to experimental media at levels less than the amount of added phosphate which is toxic in MPSS.

A small amount of growth was observed when 0.0125% yeast extract was incorporated into the "synthetic casein hydrolysate" medium; consequently, various vitamins (Table 4) were added in unsuccessful attempts to eliminate the use of yeast extract. Vitamins in various combinations were added from stock solutions after the pH of the other components of the medium had been adjusted to 6.8, and the medium was sterilized by filtration.

Occasionally some very slight turbidity was visible in flasks of the vitamin-containing media, but this growth response was not reproducible and was not correlated with the presence of any specific vitamin. These cultures never reached high turbidities, growth becoming barely visible after about 48 h and increasing only slightly by the time the cells settled to the bottom on the fourth or fifth day.

TABLE 3

Amino Acid Mixtures Employed as "Synthetic Casein Hydrolysate"

a. Amino Acid Mixture Used by Wells[1]

Amino Acid	Concentration Used in Medium (g/l)
L-alanine	0.064
L-arginine	0.082
L-aspartic acid	0.142
L-cystine	0.007
L-cysteine HCl	0.100
L-glutamic acid	0.448
glycine	0.040
L-isoleucine	0.122
L-leucine	0.184
L-lysine	0.164
L-methionine	0.056
L-phenylalanine	0.100
L-proline	0.212
L-serine	0.126
L-threonine	0.098
L-tryptophane	0.024
L-tyrosine	0.126
L-valine	0.154
L-asparagine	0.010
L-glutamine	0.010

b. Composition of Casein as listed by Bridson and Brecker (6)

L-alanine	0.060
L-arginine HCl	0.035
L-aspartic acid	0.093
L-cystine	0.008
L-glutamic acid	0.105
glycine	0.025
L-histidine	0.018
L-isoleucine	0.068
L-leucine	0.088
L-lysine	0.093
L-methionine	0.043
L-phenylalanine	0.018
L-proline	0.103
L-serine	0.240
L-threonine	0.063
L-tyrosine	0.078
L-valine	0.103

[1] M.S. thesis, Virginia Polytechnic Institute and State University, 1966.

TABLE 4

Vitamins Added to "Synthetic Casein Hydrolysate" Media in Unsuccessful Attempts to Support Growth of *S. volutans*

Vitamin	Concentration Used in Medium (mg/l)
Thiamine HCl	1
Riboflavin	1
Pyridoxine HCl	1
Pyridoxamine	1
Pyridoxal HCl	1
Calcium Pantothenate	1
Niacin	1
Niacinamide	1
L-Ascorbic Acid	1
Folic Acid	0.05
PABA	0.05
Biotin	0.005
Vitamin B_{12}	0.005
Leucovorin	0.01

The pH of one such four-day culture was measured and found to be 6.7; thus the poor growth response probably was not attributable to any wide deviation from the initial pH of 6.8. Microscopically, such cells appeared abnormally long, often attaining 15 to 20 turns of the helix, with extra fascicles of flagella at one or more points along the length where division apparently had been begun but not completed. These cells moved slowly without reversing direction, and sometimes the helix itself appeared curved or bent. Such cultures did not grow upon subculture to the same medium.

Since "synthetic casein hydrolysate" with added vitamins did not support growth when substituted for acid-hydrolyzed casein, and since a slight growth response was obtained with added yeast extract, attempts were made to obtain growth by adding various compounds which might be growth factors for S. volutans present in yeast extract or in casein hydrolysate. Supplementing the amino acids-vitamins-succinate-salts medium with purines and pyrimidines (adenine, guanine HCl, xanthine, uracil, and thymine) at concentrations of 0.1% did not produce a growth response. Tween 80, which stimulates the growth of some anaerobes (35), was tried at a concentration of 0.2% without success.

In an attempt to determine whether the growth-promoting factor in the casein hydrolysate was inorganic, a sample of casein hydrolysate was ashed at 500 C for 2 h and the residue added to the amino acids-vitamins-succinate-salts medium. No growth was obtained. Substitution of tap water for deionized water also did not permit growth in the defined medium.

Attempts were made to support a consistent, low basal level of growth in amino acids-vitamins-succinate-salts medium by adding casein hydrolysate at a very low concentration (0.01%). This medium supported good growth in 24 h when inoculated with 1 ml of a 24-h MPSS culture, but secondary serial transfers of 1 ml failed to grow. Studies were then begun to determine whether the amino acid mixture might be toxic to S. volutans. A medium consisting of 0.025% casein hydrolysate, succinate, and salts supported limited growth in the presence of one or a few amino acids (tryptophane, asparagine, glutamine), but not in the presence of the complete amino acid mixture.

In order to determine which of the amino acids in "synthetic casein hydrolysate" was (were) inhibitory, the twenty amino acids listed in Table 3a were divided arbitrarily into five groups of four amino acids. These groups were prepared as five separate solutions, adjusted to pH 6.8 with KOH, and sterilized by filtration into flasks containing sterile casein hydrolysate-succinate-salts medium (0.025% casein hydrolysate). The final concentrations of amino acids in the medium were the same as in "synthetic casein hydrolysate." The only one of these test media which did not support growth contained cysteine, glutamic acid, glycine, and isoleucine. When these four amino acids were tested separately, only cysteine inhibited growth.

"Synthetic casein hydrolysate" with cysteine omitted was not inhibitory if added to CHSS medium (0.025% casein hydrolysate), but it did not support growth in the absence of casein hydrolysate. Variation of the phosphate concentration, addition of vitamins, doubling the

concentrations of all the amino acids, and addition of trace minerals were without effect.

Throughout the process of testing experimental media, it was impossible to know whether a given compound, which failed to support growth under the experimental conditions used, might in fact be required for growth in combination with other factors not present in the test medium. It was for this reason that the trace minerals solution of Hylemon was retained as a standard component of experimental defined growth media. Although this solution did not support growth when tested, it was reasoned that it probably would do no harm, and possibly would be helpful, if it were present when other compounds were tested.

Buffering of growth media. MPSS and CHSS contain no buffers added as such; any resistance to change in pH would depend mainly on the buffering capacity of the amino acids present. Respiration of succinate by S. volutans produces a rise in pH (Caraway, B.H., doctoral dissertation, Virginia Polytechnic Institute and State University, 1972, p. 100). In order to experiment with defined growth media containing only a few amino acids at low concentrations, it was desirable to buffer the medium in order to prevent a rise in pH to inhibitory levels.

Phosphate buffer is not suitable for growth studies with S. volutans because of its toxicity to the organism (19, 37). BES buffer had been used in the DMM of Caraway and Krieg (13) and in DTM with no adverse effect on motility, although its effects on growth were unknown. BES was considered a desirable buffer for a growth medium since it is

non-metabolizable and has a pK (7.12) near the optimal pH for growth of S. volutans (6.8).

A preliminary experiment was conducted to determine the effect of BES on growth of S. volutans in CHSS medium. CHSS containing trace minerals was supplemented with BES at concentrations ranging from 5 X 10^{-4} to 10^{-1} M. Ten-ml quantities of medium in screw-capped culture tubes were inoculated with 0.1 ml of a 48-h CHSS culture and incubated in an air atmosphere. The results of this study are presented in Table 5. After 48 h of incubation, the tubes first were observed without mixing to note whether the spirilla were suspended (still swimming) or had sunk to the bottom (were non-motile). Microscopic observations after mixing confirmed this estimate of the condition of the cells as well as the purity of the cultures. The final pH of the medium was determined as an index of the effectiveness of the buffer in preventing a rise in pH. The turbidity of the three tubes which contained the most suspended cells by inspection was measured in the Klett colorimeter. From these data it was determined that 5 X 10^{-3} M BES should effectively prevent excessive changes in pH without inhibiting growth, and this concentration of BES was adopted as a standard component of experimental growth media.

In medium containing BES, "synthetic casein hydrolysate" still did not support growth. Other combinations of ingredients tested included two simplified mixtures of amino acids (mixture 1: aspartic acid, asparagine, glutamic acid, glutamine, methionine; mixture 2: aspartic acid, glutamic acid, histidine, serine). The following supplements also were

TABLE 5

Effect of Various Concentrations of BES Buffer on Growth of S. volutans in CHSS Medium[1]

Concentration of BES (M)	Macroscopic observation	Growth response,[2] 48 h	Final pH
0 (control)	Cells sunk to bottom	NT	7.48
5×10^{-4}	" " " "	NT	7.40
1×10^{-3}	No cells sunk to bottom	15	7.36
4×10^{-3}	" " " " "	23	7.12
7×10^{-3}	Some cells sunk to bottom	23	6.86
1×10^{-2}	" " " " "	NT	6.88
4×10^{-2}	" " " " "	NT	6.70
7×10^{-2}	Very sparse growth	NT	6.68
1×10^{-1}	No visible growth	NT	6.64

[1]Inoculum consisted of 0.1 ml of a 48-h culture in CHSS medium.

[2]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values are averaged from duplicate tubes. Organisms were cultured in 10-ml quantities of medium contained in 20 X 125 mm tubes with screw caps loosened. NT = not tested.

added experimentally to amino acid media: 0.1% ammonium acetate; 0.1% (v/v) lactic acid; 0.01 M sodium formate; and $CaCl_2$ at concentrations ranging from 5×10^{-4} M to 1×10^{-2} M, with or without 0.1% NaCl. Phosphate concentrations were varied in several of these media over a concentration range of 10^{-4} M to 10^{-1} M. In all cases, no growth occurred.

Analysis of casein hydrolysate. When no attempt to devise a "synthetic casein hydrolysate" growth medium had been successful, attention was turned to study of the acid-hydrolyzed casein in order to learn more about the nature of the component(s) which supported growth. Extraction of a 2.5% solution of casein hydrolysate with an excess of ether before incorporation into CHSS medium did not affect growth. When CHSS medium was dialyzed in the cold against an equal volume of deionized water for 22 h, the fractions from both sides of the dialysis membrane supported growth equally well.

Gas chromatography of an ether extract of an acidified 10% solution of casein hydrolysate showed that no detectable alcohols or volatile fatty acids were present. When the sample was methylated and extracted with chloroform, a very small amount of oxalic acid and a questionable trace of pyruvic and lactic acids were detected. These small amounts were considered probably insignificant in view of the fact that the concentration of casein hydrolysate in which they were detected was 40 times that routinely used in CHSS.

The "growth-promoting factor" evidently was not heat-labile, as CHSS medium routinely was sterilized by autoclaving. In addition, a

casein hydrolysate stock solution was autoclaved for 1 h at 121 C and incorporated into CHSS medium, and this medium supported growth equal by turbidimetric measurement to that obtained in CHSS made with casein hydrolysate which had not been autoclaved.

Stimulation experiments. Because the approach of making the synthetic medium as complete as possible had proved unproductive, it was decided to test individual compounds for any stimulatory effect on growth. A low basal level of growth was obtained in CHSS by decreasing the casein hydrolysate content to 0.1%; the medium was dispensed in 5-ml amounts into 20 X 125 mm tubes having loosened screw caps. The effect on growth of any added compound was determined turbidimetrically.

Because the tubes were to be incubated in an air atmosphere, the possibility of including an agent to lessen the toxic effects of peroxides was considered, especially in view of the low catalase activity reported previously for S. volutans (19, 38). Addition of 0.2 mg (740 units) per ml of bovine liver catalase to CHSS medium was attempted, but a fine precipitate formed during incubation of this medium. The precipitated material appeared microscopically as small globules; in shaken cultures the material aggregated into a soft, brownish mass. The precipitation occurred whether or not the medium had been inoculated. Omitting succinic acid, ammonium sulfate, or magnesium sulfate from the medium did not eliminate formation of the precipitate. No stimulation of growth by catalase was observed by macroscopic or microscopic inspection, and turbidimetric measurement of growth was impossible because of the precipitate.

Pyruvate has been reported (59) to react spontaneously with H_2O_2, forming acetate, H_2O, and CO_2. However, pyruvic acid at 0.1% concentration completely inhibited growth of S. volutans in casein hydrolysate medium (0.1% casein hydrolysate + succinate-salts).

Hemin was tested because of the possibility that it might enhance catalase production or otherwise stimulate growth as it does with some anaerobes (35). The hemin was prepared by the method recommended in the VPI Anaerobe Laboratory Manual (35) except that KOH, rather than NaOH, was used as the solvent. Hemin at a concentration of 0.001% was not inhibitory, but neither did it enhance growth. It had a tendency to precipitate and interfere with turbidity measurements, and its use was abandoned.

Amino acids, vitamins, purines, pyrimidines, and tricarboxylic acid cycle intermediates were tested for stimulatory activity by adding these compounds to casein hydrolysate-succinate-salts medium containing 0.1% casein hydrolysate. The results of one experiment are presented in Table 6. From this and other experiments, five of more than thirty compounds tested were selected as being stimulatory. These were tyrosine, cystine, and lysine (0.05%); oxalacetic acid (0.1%); and guanine (0.01%).

These five stimulatory compounds, when used together at the concentrations listed above, did not support growth when substituted for casein hydrolysate in CHSS.

An extensive series of test media was then prepared, using as a base succinate-salts with trace minerals, BES buffer, and 5×10^{-3} M K_2HPO_4. The five compounds mentioned above were added to this base

TABLE 6

Stimulation of Growth of S. volutans by Various Compounds in the Presence of Casein Hydrolysate[1]

Compound tested	% Concentration	Turbidity[2] at 24 h	Compound tested	% Concentration	Turbidity[2] at 24 h
None (control)	-----	11	L-Serine	0.05	15
L-Alanine	0.05	14	L-Threonine	"	17
L-Arginine	"	17	L-Tryptophane	"	14
L-Asparagine	"	16	L-Tyrosine	"	42
L-Aspartic Acid	"	18	L-Valine	"	12
L-Cystine	"	60	L-Malic Acid	0.01	15
L-Glutamic Acid	"	13	L-Malic Acid	0.04	13
L-Glutamine	"	15	Oxalacetic Acid	0.01	8
Glycine	"	6	Oxalacetic Acid	0.10	56
L-Histidine	"	16	Adenine	0.01	11
Hydroxy-L-Proline	"	14	Guanine	0.01	precipitate[3]
L-Isoleucine	"	16	Thymine	0.01	13
L-Leucine	"	14	Uracil	0.01	21
L-Methionine	"	15	Niacin	0.0001	14
L-Phenylalanine	"	11	Niacinamide	0.0001	12
L-Proline	"	15	Yeast Extract	0.01	20

[1] Medium contained succinate-salts, trace minerals, BES, and 0.1% casein hydrolysate. The inoculum consisted of 2 drops of a 75-Klett unit suspension of cells from a 24-h MPSS culture, washed once in and resuspended in DMM.

[2] Klett units, using 16-mm cuvettes and blue (420 nm) filter. Values are averaged from duplicate tubes. Organisms were cultured in 5-ml quantities of medium contained in 20 X 125 mm tubes with screw caps loosened.

[3] A precipitate formed which prevented turbidimetric measurement; but good growth was present as determined by microscopy.

singly or in various combinations. No growth occurred when all five of these compounds were present simultaneously, regardless of the presence of 0.1% casein hydrolysate, 0.01% yeast extract, the "11 amino acid mixture" (see Materials and Methods), 0.01% calcium lactate, or 0.01% uracil in various combinations. Growth did occur when 0.15% purified agar was incorporated into a medium containing the 11 amino acid mixture, 0.01% calcium lactate, and 0.01% uracil in addition to the tyrosine, cystine, lysine, oxalacetate, and guanine; but an identical medium without agar did not support growth. Stimulation of growth in medium with casein hydrolysate was evident when the stimulatory compounds were present in various combinations of two (43-63 Klett units, compared with 30 Klett units for 0.1% casein hydrolysate control). Growth in medium without casein hydrolysate, however, occurred in only one case, as described in the following section.

Growth in medium containing autoclaved tyrosine. It should be mentioned at this point that, although all amino acid stock solutions had been sterilized by filtration, the 0.5% tyrosine stock did not remain in solution on standing, and therefore it was redissolved by boiling prior to each use. Boiling frequently was accomplished by steaming in the autoclave for a few minutes with the vent open. The pH of the solution was about 9, the tyrosine having been dissolved originally in 2 N KOH and brought to volume with water. The solution became increasingly yellow when subjected repeatedly to this treatment.

The first instance of growth of S. volutans in a "defined" medium occurred in a medium of the following composition: succinate-salts,

trace minerals, 5×10^{-3} M BES, 5×10^{-3} M K_2HPO_4, 11 amino acid mixture, 0.01% calcium lactate, and 0.05% tyrosine from the repeatedly boiled stock solution described above. This medium supported growth of a washed inoculum of S. volutans to a turbidity of 42 Klett units, compared to 30 Klett units in the control with 0.1% casein hydrolysate.

As soon as growth had been obtained in a "defined" medium containing tyrosine, attempts were made to transfer this culture in a medium prepared from the same stock solutions, and also to repeat the experiment using freshly-prepared stock solutions. These attempts were uniformly unsuccessful, despite variations in the phosphate concentration and pH and also addition of cystine, lysine, oxalacetate, or activated charcoal (Norit). The tyrosine medium supported growth only if casein hydrolysate, yeast extract, or purified agar was added, and only the casein hydrolysate supported growth beyond the first transfer.

Growth finally was obtained after 48 h of incubation in a medium containing the components listed in Table 7 and designated the "initial defined medium." The tyrosine and cystine in this medium came from a mixed stock solution of 1% tyrosine: 0.1% cystine, dissolved in KOH, which had been sterilized by filtration and redissolved once by steaming. The pH of the stock solution was 9.

The growth which appeared after 48 h of incubation in the above medium was present in duplicate tubes under both air and 6% O_2: 94% N_2 atmospheres. When these cultures were transferred to several variations of the same medium, it was discovered that growth was greatly enhanced by the addition of 0.0085% NaCl to the medium.

TABLE 7

Composition of the Initial Defined Medium

Compound[1]	Amount (g/l)	Compound[1]	Amount (g/l)
2 Succinic acid	1.0	L-tyrosine	0.125
2 $MgSO_4 \cdot 7H_2O$	1.0	L-cystine	0.0125
2 $(NH_4)_2SO_4$	1.0		
2 $FeCl_3 \cdot 6H_2O$	0.002	4 L-lysine	0.2
2 $MnSO_4 \cdot H_2O$	0.002	4 L-threonine	"
		4 L-histidine	"
3 $CaCO_3$	0.001	4 L-serine	"
3 $ZnSO_4 \cdot 7H_2O$	0.00072	4 L-isoleucine	"
3 $CuSO_4 \cdot 5H_2O$	0.000125	4 L-arginine	"
3 $CoSO_4 \cdot 7H_2O$	0.00014	4 L-asparagine	"
3 H_3BO_3	0.000031	4 L-aspartic acid	"
3 $Na_2MoO_4 \cdot 2H_2O$	0.000245	4 L-glutamine	"
		4 L-glutamic acid	"
Oxalacetic acid	0.1	4 L-methionine	"
Calcium lactate	0.1		
Vitamin B_{12}	1×10^{-7}	BES	1.07
PABA	2×10^{-4}	K_2HPO_4	0.87
		NaCl[5]	0.085

[1]Medium was prepared by combining stock solutions which had been sterilized by filtration, except succinate-salts, which was autoclaved.

[2]Succinate-salts

[3]Trace minerals

[4]11 amino acid mixture

[5]Not present when growth first obtained, but subsequently found stimulatory.

Cultures of S. volutans were carried through six serial transfers using one drop of each previous culture as inoculum for 5-ml quantities of the above medium in screw-capped tubes under an air atmosphere.

Attempts to reproduce this work and to eliminate some components from the initial defined medium, however, soon revealed an erratic pattern in which one-drop inocula from 24-h "defined medium" cultures might grow after 24 or 48 h, or might not grow at all. When no growth was obtained, usually reinoculation with three drops, or with one drop from a very turbid culture in "defined medium," would produce growth.

An attempt to prepare the "defined medium" by weighing and mixing all of the components, without use of the previously prepared stock solutions, resulted in no growth in any of 28 tubes which included duplicate tubes of complete medium and of 13 variations, such as leaving out each amino acid in turn or substituting oxalate or pyruvate for the oxalacetate in the medium.

After this attempt, new stock solutions of the medium components were prepared and combined to reconstruct the medium, but this medium also failed to support growth. These results suggested that some growth-stimulating impurity might be present in one or more of the original stock solutions. In order to locate the source of the activity, the old stock solutions were substituted, one at a time, in media otherwise prepared from new stock solutions. Excellent growth was obtained in the medium containing the old stock of tyrosine and cystine, which had been redissolved by steaming before each use. None of the other old stock solutions supported growth when used in new medium.

Consideration of the insolubility of tyrosine and of the harsh treatment which had been used to dissolve it suggested the possibility that heat treatment of tyrosine under alkaline conditions produces some breakdown product which is a growth factor for S. volutans. This possibility was tested in the following manner. A new solution of 1% tyrosine: 0.1% cystine was prepared by dissolving these amino acids in 2 N KOH, diluting with water, and lowering the pH to 9.2 with 2 N HCl. Brief boiling was necessary to dissolve the tyrosine and cystine completely. This stock solution was divided into two parts, one of which was sterilized by filtration while the other one was autoclaved at 121 C for 15 min. Only the autoclaved solution supported growth of S. volutans in the "defined" medium.

That the significant factor was the altering of the tyrosine by autoclaving, and not destruction or alteration of cystine, or interaction between the two, was demonstrated by the following experiments:

1. If the cystine concentration in the medium was lowered 10-fold (to 0.000125%), but both tyrosine and cystine were sterilized by filtration, the medium did not support growth. This fact was evidence against the cystine concentration being too high initially and partially destroyed by autoclaving.

2. If the tyrosine and cystine solutions were autoclaved separately, the medium supported growth. Therefore, interaction between the two compounds in the autoclave was not necessary.

3. If the tyrosine solution was autoclaved and the cystine solution sterilized by filtration, the medium still supported growth, al-

though no growth was obtained if either the autoclaved tyrosine or the cystine was omitted.

A nutritional requirement for autoclaved tyrosine presumably would be a requirement for some breakdown product formed by heat treatment at pH 9. The nature of this breakdown product and its nutritional role was a mystery, and some work was undertaken to try to determine what it might be.

One possible mode of degradation of tyrosine (p-hydroxyphenylalanine) in the autoclave might be by deamination, producing p-hydroxyphenylpyruvic acid. An attempt was made to substitute the latter for autoclaved tyrosine, at a concentration of 0.0125%, in the initial defined medium. p-Hydroxyphenyl pyruvic acid did not support growth, but it was not inhibitory because growth did occur when autoclaved tyrosine also was present.

Paper chromatography of autoclaved tyrosine. Paper chromatography of filtered and autoclaved tyrosine solutions was used in an attempt to distinguish any breakdown products which might be formed during autoclaving. The tyrosine was chromatographed by the two procedures described in Materials and Methods. Only one spot appeared on such chromatograms. Using these methods, no difference could be discerned between autoclaved tyrosine and tyrosine which had not been autoclaved.

Relationship of autoclaved tyrosine to iron metabolism. The search for a metabolically useful breakdown product of tyrosine was materially aided by the discovery of a report by Gorini and Lord (25), who had studied a bacterium (Micrococcus sp.) which required autoclaved tyrosine

for growth in a defined medium. They traced the activity to the occurrence of ortho-dihydroxyphenyl compounds, which according to Hutner (36) serve as chelators to facilitate solubilization of ferric ions for transport into the cell.

Replacement of autoclaved tyrosine by ferrous iron. The succinate-salts mixture used in the defined medium for S. volutans contained 2 mg/l $FeCl_3 \cdot 6H_2O$. At pH 6.8, very little of this ferric iron can be expected to be in solution; the optimum pH for solubility of ferric ions is near 5 (43). In an attempt to provide a more available form of iron, an experimental defined medium containing $FeSO_4 \cdot 7H_2O$ at a concentration of 30 mg/l, and no ferric iron, was prepared. The concentration of ferrous sulfate used was suggested by Hutner and is equivalent to 6 mg/l of ferrous iron. The medium supported excellent growth of S. volutans through at least three serial transfers in the absence of any tyrosine, autoclaved or otherwise. However, a white precipitate formed in the medium during incubation and interfered with turbidimetric measurement of growth. Good growth, as determined by visual inspection of the band of cells which formed just below the surface, occurred in medium containing up to 90 mg $FeSO_4 \cdot 7H_2O$/l, but higher levels were increasingly inhibitory. No growth was observed when the level of ferrous sulfate was raised to 300 mg/l or lowered to 3 mg/l.

Use of artificial chelators for ferric iron. Gorini and Lord (25) listed a number of o-dihydroxyphenyl compounds which were effective in promoting the growth of their micrococcus, although it was Hutner (36)

who attributed the efficacy of these compounds to their ability to chelate iron. In the present work, the effect of several such compounds was studied in S. volutans. The defined basal medium (DBM) used in these studies was that presented in Table 13; development of other aspects of this medium (amino acid composition, etc) will be discussed later.

In the initial study, 10^{-5} M catechol, 10^{-5} M pyrogallol, and 2×10^{-5} M DL-arterenol (DL-norepinephrine) were tested in DBM. Two levels of ferric iron were employed, 30 mg/l and 3 mg/l $FeCl_3 \cdot 6H_2O$ (approximately equivalent to 6 mg/l and 0.6 mg/l as Fe^{+++}, respectively). The medium was prepared by combining sterile stock solutions, and cotton-plugged tubes containing 5-ml amounts of medium were inoculated with one drop of a culture grown in DBM with $FeSO_4$ (30 mg/l) instead of ferric iron.

Control tubes containing no chelating agent supported limited growth, but no growth appeared in the second serial transfer at the lower level of $FeCl_3$, and the culture with the higher iron level died out after three transfers made at 24-h intervals. Catechol and pyrogallol at 10^{-5} M appeared to be toxic, as no growth occurred in these media even in the first transfer. Media containing 2×10^{-5} M DL-arterenol, however, supported good growth, as shown by Table 8, which presents the turbidity readings in the third serial transfer. As shown by the table, better growth in the presence of arterenol was obtained when the lower $FeCl_3$ concentration of 3 mg/l was used. Media containing arterenol with the higher iron level (30 mg/l) developed a blue color during incubation.

TABLE 8

Growth-stimulating Effect of DL-Arterenol on S. volutans in DBM[1] Containing Two Levels of $FeCl_3 \cdot 6H_2O$

Concentration of DL-Arterenol (M)	Concentration of $FeCl_3 \cdot 6H_2O$ (mg/l)	Growth response[2] at 24 h in third serial transfer
2×10^{-5}	3	35
2×10^{-5}	30	15
0	3	0
0	30	0

[1]For composition of DBM, see Table 13.

[2]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values are averaged from duplicate tubes. Organisms were cultured in 5-ml quantities of medium contained in 20 X 125 mm cotton-plugged tubes. The inoculum consisted of one drop of a culture grown in DBM containing $FeSO_4$ (30 mg/l) instead of ferric iron.

It became apparent that the ability to grow without chelators was related to inoculum size. When one drop of a culture grown with $FeSO_4$ was transferred to DBM containing $FeCl_3$ without arterenol, growth was initiated after a long lag period; cultures with no visible growth at 24 h sometimes yielded a growth response of 35 Klett units at 36 h. The presence of a chelating compound such as arterenol was stimulatory to such cultures but was necessary for growth when smaller inocula were used. Cultures which initiated growth in chelator-free media could be transferred successfully after growth became heavy, but if transferred after 24 h they failed to grow unless a chelator was added. The stimulatory effect of DL-arterenol was clearly demonstrated by an experiment in which a tube of DBM containing 3 mg/l ferric chloride, but no arterenol, was inoculated with one drop of a culture grown with ferrous sulfate (30 mg/l). Two subcultures were then made from this tube, after 24 and 48 h of incubation, by transferring one drop into duplicate tubes of identical medium with and without 10^{-5} M DL-arterenol.

The first of these subcultures was not able to grow without arterenol and yielded a growth response of only 8 Klett units after 60 h of incubation when its counterpart with arterenol measured 70 Klett units. However, the second subculture, made at 48 h, grew to a turbidity of 36 Klett units in 36 h of incubation. Arterenol still was stimulatory, producing a turbidity of 45 Klett units in 36 h (all values are averages of duplicate tubes).

A study was undertaken to determine whether some of the other o-dihydroxyphenyl compounds mentioned by Gorini and Lord (25) would stimulate growth of S. volutans in DBM containing 3 mg/l ferric chloride.

The compounds initially were tested at 10^{-5}, 10^{-6}, and 10^{-7} M (in the concentration range used by Gorini and Lord). Duplicate tubes were inoculated with one drop of a 24-h culture grown in DBM with ferrous sulfate. As shown by the results in Table 9, an inoculum of this size was able to initiate growth in the absence of an iron chelator. However, the first seven dihydroxyphenyl compounds listed (all enantiomers of DOPA, arterenol, or epinephrine) stimulated growth by approximately 20% when incorporated into the medium at concentrations of 10^{-5} or 10^{-6} M. Those cultures containing only 10^{-7} M chelators had turbidities similar to or less than that of the control. Protocatechuic acid at these concentrations did not appear to affect growth. As determined in the earlier experiment described above, catechol and pyrogallol at 10^{-5} M were decidedly inhibitory. These compounds produced only slight stimulation at 10^{-6} M, but this effect again was absent at 10^{-7} M.

When subcultures were made from the control and all cultures containing 10^{-6} M dihydroxyphenyl compounds, by transferring one drop of each 48-h culture into homologous medium, the results after 36 h of incubation were as shown in Table 10. However, the entire effect is not reflected in the turbidities at 36 h, because the controls without dihydroxyphenyl chelators could eventually grow to high densities but exhibited a very long lag period. When observed at 24 h, very slight turbidity was visible in only one duplicate of the control culture, and no visible growth in the other; good growth was apparent at that time in all cultures containing chelators. Therefore, most of the

TABLE 9

Effect of Various Concentrations of o-Dihydroxyphenyl Compounds on Growth from a Large Inoculum[1] of S. volutans in DBM[2]

Compound added to DBM	Growth response[3] at 48 h with the following concentrations (M):			
	0	10^{-5}	10^{-6}	10^{-7}
None (control)	56			
L-DOPA		69	68	54
D-DOPA		65	65	56
L-Arterenol		65	67	57
DL-Arterenol		64	68	52
L-Epinephrine		65	65	61
DL-Epinephrine		67	67	61
Protocatechuic Acid		58	58	55
Catechol (Pyrocatechol)		46	61	58
Pyrogallol		16	64	47

[1]Inoculum = 1 drop of a 24-h culture grown in DBM containing 30 mg/l $FeSO_4 \cdot 7H_2O$ instead of 3 mg/l $FeCl_3 \cdot 6H_2O$.

[2]For composition of DBM, see Table 13.

[3]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values are averaged from duplicate tubes. Organisms were cultured in 5-ml quantities of medium contained in 20 X 125 mm tubes with loosened screw caps.

TABLE 10

Growth of Second-transfer Cultures[1] of S. volutans in DBM[2] with and without 10^{-6} M o-Dihydroxyphenyl Compounds

Compound added to DBM (10^{-6} M)	Growth Response[3] (36 h)
None (control)	36
L-DOPA	45
D-DOPA	49
L-Arterenol	48
DL-Arterenol	45
L-Epinephrine	50
DL-Epinephrine	49
Protocatechuic Acid	40
Catechol	43
Pyrogallol	52

[1]Inoculum = 1 drop of 48-h culture in homologous medium.

[2]For composition of DBM, see Table 13.

[3]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values are averaged from duplicate tubes. Organisms were cultured in 5-ml quantities of medium contained in 20 X 125 mm tubes with loosened screw caps.

growth attained in the control cultures occurred during the last 12 h of incubation.

As shown in Table 10, the difference in turbidity between the control culture and any one of the cultures with dihydroxyphenyl compounds is not outstanding; but taken together, the data show that all cultures containing dihydroxyphenyls achieved final cell densities somewhat higher than that of the control. Statistical analysis by the t-test indicates that the cultures with added iron chelators, taken as a group, had significantly higher turbidities than the control cultures ($p < 0.01$)*.

Lankford (43) has discussed the relationship of "minimum inoculum size" and "inoculum-dependent lag" to the time required for inoculum cells to produce iron chelators in sufficient concentration for growth initiation (see Review of the Literature). It seemed possible that S. volutans produces small amounts of an iron-chelating compound at a rate sufficient to allow initiation of growth only from larger inocula unless an exogenous chelator is supplied. In order to determine the size of the minimum inoculum capable of initiating growth in DBM with 3 mg/l of $FeCl_3 \cdot 6H_2O$ and no exogenous chelator, the experiment summarized in Table 11 was performed. Growth, as measured by turbidity after 45 h of incubation, occurred in the absence of exogenous chelators from an inoculum of 3.2×10^4 washed cells/ml, but not from a twofold dilution of this inoculum. Growth was obtained from 1.7×10^4 cells when 10^{-6} M L-epinephrine was present in the medium.

* $t_{obt} = 3.089$; $t_{.01}$ (18 df) = 2.88.

TABLE 11

Effect of Size of Inoculum on Growth of S. volutans in DBM[1] Containing L-Epinephrine and/or S. volutans Culture Filtrate

Additions to DBM	Inoculum size (cells per ml of culture medium)	Growth response[2], 45 h
None	8.5×10^3	0
	1.7×10^4	0
	3.2×10^4	28
5% (v/v) of culture filtrate[3]	8.5×10^3	0
	1.7×10^4	15
	3.2×10^4	22
10^{-6} M L-epinephrine	8.5×10^3	0
	1.7×10^4	39
	3.2×10^4	39
10^{-6} M epinephrine + 5% (v/v) culture filtrate	8.5×10^3	13
	1.7×10^4	36
	3.2×10^4	35

[1]For composition of DBM, see Table 13.

[2]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values are averaged from duplicate tubes. Organisms were cultured in 5-ml quantities of medium contained in 20 X 125 mm tubes with loosened screw caps.

[3]Culture filtrate was prepared from 47-h-old cultures of S. volutans grown in DBM containing 30 mg/l $FeSO_4 \cdot 7H_2O$ instead of ferric chloride.

Some preliminary work with a culture filtrate also was included in this experiment (Table 11). The filtrate was prepared from a 47-h-old culture of S. volutans grown in DBM containing 30 mg $FeSO_4 \cdot 7H_2O$ / l and no dihydroxyphenyl compounds. The incorporation of 5% (v/v) of this filtrate into DBM permitted growth from 1.7 X 10^4 cells/ml, as did epinephrine. Filtrate and epinephrine together allowed growth from an inoculum half this size.

To summarize this section, o-dihydroxyphenyl compounds were stimulatory to large inocula but were critical to initiation of growth if the inoculum was small. These observations may explain the erratic results obtained earlier with autoclaved tyrosine, when growth frequently was obtained by reinoculating media which initially had failed to support growth.

Amino acid requirements. The initial defined medium (Table 7) contained several components which were not known to be necessary. Growth was found to occur readily on removal of calcium lactate, vitamin B_{12}, and oxalacetate. The small amount (0.0085%) of NaCl was found to be required for growth, as was cystine. The autoclaved tyrosine was replaced by any of a number of o-dihydroxyphenyl iron chelators.

The "11 amino acid mixture" was studied in order to determine which, if any, of its components were required for growth. The first step in this study was to prepare the eleven amino acids as separate stock solutions and to test the growth response of S. volutans in defined medium lacking each amino acid singly. The results of this experiment are presented in Table 12, which shows that growth was

TABLE 12

Effect on Growth of S. volutans when Each of Eleven Amino Acids was Omitted Singly from the Initial Defined Medium[1]

Amino acid omitted	Growth response[2], 42 h
None (control)	39
L-Lysine[3]	40
L-Threonine[3]	24
L-Histidine[3]	31
L-Serine	39
L-Isoleucine[3]	33
L-Arginine	45
L-Asparagine	39
L-Aspartate	52
L-Glutamine	41
L-Glutamate	46
L-Methionine[3]	29

[1]The composition of the initial defined medium is given in Table 7. Calcium lactate, vitamin B_{12}, and oxalacetate had already been eliminated from the medium when these experiments were done. The inoculum consisted of one drop from a 24-h-old culture grown in initial defined medium with the above modifications but containing the complete 11 amino acid mixture.

[2]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. The values given were averaged from duplicates. Organisms were cultured in 5-ml quantities of medium contained in 20 X 125 mm tubes with loosened screw caps.

[3]Amino acids included in final composition of DBM.

considerably less than the control level when either threonine or methionine was omitted from the medium. When each culture was transferred in homologous medium, barely visible turbidity was obtained without either threonine or methionine, but the other cultures grew well.

A defined medium containing cystine, threonine, and methionine as the only amino acids, however, supported growth for only one transfer. Continued growth on subculture was obtained by adding histidine and isoleucine to the simplified medium. These amino acids were chosen because they were the only ones besides threonine and methionine which, when omitted, caused any reduction in the amount of growth obtained compared to a control containing all eleven amino acids (Table 12).

The defined medium at this point contained cystine, which was known to be required, and four other amino acids. Further experimentation with these four revealed that growth in at least two serial subcultures could be obtained when any one of them, except histidine, was omitted; medium without histidine did not support growth. Medium containing histidine and any one of the other three amino acids supported growth to approximately equal turbidity (average = 21 Klett units), but an improvement of about 50% in the amount of growth was noted when all four amino acids were present (average = 32 Klett units). Therefore, threonine, histidine, methionine, and isoleucine were retained as standard components of the medium.

The composition of the defined basal medium (DBM) finally adopted for cultivation of S. volutans is presented in Table 13. This medium,

TABLE 13

Composition of Defined Basal Medium[1]

Compound	Amount (g/l)
Succinic acid	1.0
$MgSO_4 \cdot 7H_2O$	1.0
$(NH_4)_2SO_4$	1.0
$MnSO_4 \cdot H_2O$	0.002
K_2HPO_4	0.0174
NaCl	0.085
$CaCO_3$	0.001
$ZnSO_4 \cdot 7H_2O$	0.00072
$CuSO_4 \cdot 5H_2O$	0.000125
$CoCO_4 \cdot 7H_2O$	0.00014
H_3BO_3	0.000031
$Na_2MoO_4 \cdot 2H_2O$	0.000245
L-Threonine	0.2
L-Histidine	0.2
L-Methionine	0.2
L-Isoleucine[2]	0.2
L-Cystine[3]	0.025
BES	1.0665
$FeCl_3 \cdot 6H_2O$[3]	0.003

[1]A basal medium to which a dihydroxyphenyl compound (usually 10^{-5} M arterenol or epinephrine) was added to support growth of S. volutans. The pH was adjusted to 6.8 with KOH.
The medium was sterilized by autoclaving (121 C for 15 min; dihydroxyphenyls added aseptically afterward) or by filtration.

[2]Isoleucine was dissolved in a small amount of 2 N HCl before adding to the medium.

[3]Cystine was dissolved in a small amount of 2 N KOH and added during late stages of pH adjustment of the medium.

[4]Ferric chloride was added aseptically after autoclaving, if the medium was autoclaved.

with arterenol or epinephrine added, supported growth consistently in daily use over a period of two months. The medium supported growth of both the Wells strain (ATCC 19554) and the Pringsheim strain (ATCC 19553) of S. volutans, and it was effective either when prepared from individual concentrated stock solutions or when the components were weighed directly and combined. Sterilization was accomplished either by autoclaving at 121 C for 15 min or by filtration through a 0.45-μ membrane filter, and the method of sterilization did not affect the growth response. Studies of the effects of o-dihydroxyphenyl iron chelators, presented in the previous section, were carried out using this medium. DBM with a chelator for ferric iron added was designated "defined growth medium" (DGM). L-arterenol or L-epinephrine (10^{-5} M) was usually employed as an iron chelator.

Studies Related to the Microaerophilism of S. volutans

A major reason for my interest in the nutrition of S. volutans was its possible relation to the microaerophilic nature of the organism. Several possible contributing causes of a requirement for low oxygen tension were investigated during the course of the nutritional work, utilizing both complex and defined media.

Effect of growth medium on oxygen tolerance. As reported by Wells and Krieg (68), S. volutans was microaerophilic in nutrient broth and in PSS medium; i.e., growth from small inocula was initiated in an atmosphere of 6% O_2: 94% N_2, but not in air.

When CHSS medium was developed, it was noted that S. volutans tolerated oxygen much better in this medium than in MPSS, as demon-

strated by the data presented in Table 14. In this experiment, a turbidity of 36 Klett units was achieved in CHSS after 48 h on a reciprocal shaker, as compared with only 7 Klett units in MPSS. This table also shows that apparently the presence of casein hydrolysate, rather than the absence of peptone, was responsible for the increased oxygen tolerance, because good growth occurred under air in "PCHSS," a medium constructed by adding both 0.5% peptone and 0.25% casein hydrolysate to the succinate-salts base.

CHSS medium was adopted as the standard growth medium for cells to be used in the bioindicator study (Results, part 1). Cultures for that study were maintained for 18 months in 80-ml quantities of CHSS by daily serial transfer of 1.5 ml. These cultures were incubated statically in cotton-stoppered flasks in an air atmosphere.

The defined growth medium, tubed in 5-ml quantities in 20 X 125 mm tubes plugged with cotton or closed loosely with screw caps, supported growth in an air atmosphere through 60 transfers in which inocula consisting of 1 drop from the previous culture were used. Growth also occurred readily when the tubes were incubated in a nearly horizontal position (to provide maximum surface area exposed to air). Cells from these cultures were shown to be still microaerophilic when transferred to MPSS medium, in which turbidity from a one-drop inoculum was achieved after 24 h in tubes containing 5 ml under a 6% O_2: 94% N_2 atmosphere, but no visible turbidity was present even after 48 h of incubation in an air atmosphere with the tubes either upright or slanted.

DGM did not, however, permit unrestricted growth under more

TABLE 14

Comparison of Growth of S. volutans in MPSS Broth, CHSS Broth, and CHSS Broth Containing 0.5% Peptone

Medium	Growth response[1] at	
	24 h	48 h
MPSS broth	7	7
CHSS broth	2	36
CHSS broth + 0.5% peptone	23	41

[1]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values represent averages from duplicate tubes. Test media (75 ml) were contained in cotton-stoppered 250-ml Erlenmeyer flasks, and cultures were incubated at 30 C on a reciprocating shaker at 82 oscillations/min. The inoculum per flask consisted of 0.25 ml of a 70-Klett unit cell suspension prepared by centrifuging a 24-h culture grown in MPSS and resuspending the cells in MPSS.

rigorous aerobic conditions (shaken cultures) despite numerous attempts. Inocula of the minimum size required for growth in unshaken tubes of defined medium (about 2 X 10^4 cells/ml) failed to grow within 48 h in 250-ml flasks containing 50 ml of medium and incubated on a reciprocating shaker (82 oscillations/min through a 3.5-cm path). Growth also failed to occur when the concentration of $FeCl_3 \cdot 6H_2O$ was increased from 3 to 30 mg/l or when the iron chelator (epinephrine in this case) was increased from 10^{-5} to 10^{-4} M. Medium with the higher concentration of epinephrine turned red during incubation.

Inocula of 7 X 10^4 washed cells/ml, or inocula consisting merely of 0.5 ml of a 24-h culture in defined medium, were found to initiate growth in shaken flasks of DGM. However, in four separate experiments, these cultures reached a light turbidity of 26-28 Klett units after 24 h of incubation and then stopped growing, producing no further increases in turbidity up to 40 h. Periodic polarographic measurement of the dissolved oxygen in the medium during the first 24 h showed it to be the same as that of uninoculated medium incubated on the same shaker. Therefore, the inoculum cells did initiate growth without first lowering the concentration of dissolved oxygen, but growth ceased prematurely. Microscopic examination at 40 h showed most of the cells to be nonmotile.

Efforts were made to obtain continued growth in such cultures by periodic additions of extra dihydroxyphenyl compounds (epinephrine or DOPA) or of succinate. Also, preparation of the original medium with $FeSO_4$ instead of $FeCl_3$ was tried, and efforts were made to retard its

oxidation by embedding it in a layer of purified agar at the bottom of the flask. However, none of these attempts were successful; the cultures either failed to grow or stopped growing after a light turbidity had been reached. Occasionally, such cultures were observed to produce heavy growth when removed from the shaker after three days and left on the bench for about a week. This growth consisted of pure, motile cultures of S. volutans as determined by darkfield microscopy.

In one series of experiments, involving 16 flasks, the defined medium was supplemented with 0.01% oxalacetate, 0.01% calcium lactate, and/or 10^{-10} g/ml of vitamin B_{12}, singly or in various combinations. (These were components of an early version of the defined medium but had been eliminated as unnecessary for growth in tubed media.) These flasks, with inocula ranging from 0.5 to 1.0 ml of dense cultures grown in DGM, showed at most only a very slight turbidity after 48 h of shaking. However, they were left on the shaker, and all of the media (regardless of the various additions) produced heavy growth (as observed visually but not quantified) between day 5 and day 9.

No definitive explanation is available at present for the erratic behavior of S. volutans when shaken in air. Shaking, per se, is not the significant factor, because control cultures shaken in sidearm flasks containing an atmosphere of 6% O_2: 94% N_2 invariably grew to high turbidities within 24 h.

S. volutans was tested also for colony formation on plates of DGM + 1.5% agar, in view of the inability of the organism to form colonies on complex agar media even under microaerophilic conditions (55, 68).

Plates of DGM (containing 10^{-5} M epinephrine) were streaked and incubated in an air atmosphere or in a candle jar, as well as in a sealed jar containing 6% O_2: 94% N_2. No growth was obtained after 5 days of incubation.

Effect of inoculum size on oxygen tolerance. Besides the differences in oxygen tolerance observed in various media, the major factor which appeared to influence the ability of S. volutans to initiate growth in any medium in an air atmosphere was the size of the inoculum. The fact that smaller inocula in defined medium required exogenous iron chelators has already been mentioned (see Table 11). In addition, inoculum size was critical to the initiation of growth in MPSS medium if a special gas atmosphere was not provided. A one-drop inoculum in a flask containing 75 ml of MPSS grew as well as a 1-ml inoculum if both were incubated under 6% O_2; but the one-drop inoculum produced no visible turbidity in 24 h if incubated in an air atmosphere, even without shaking.

Table 15 illustrates the effect of inoculum size on growth of S. volutans in MPSS medium with agitation. In an air atmosphere, the turbidity after 23 h incubation was directly proportional to the size of the inoculum. In a 6% oxygen atmosphere, however, growth was readily initiated from small inocula, as shown by the fact that a 0.1-ml inoculum grew to 50 Klett units in 29 h under microaerophilic conditions, while identical cultures incubated in air measured only 3 Klett units.

Inocula consisting of 1.0 ml of a 70-Klett unit suspension did not, however, always produce growth in MPSS medium incubated in air with

TABLE 15

Effect of Inoculum Size on Initiation of Growth of S. volutans in MPSS under an Air Atmosphere

Inoculum size[1] (ml)	Atmosphere[2] in which shaken	Growth response[3] at:	
		23 h	29 h
0.1	6% O_2: 94% N_2	14	50
0.1	Air	4	3
0.25	"	12	12
0.5	"	22	ND
1.0	"	50	ND

[1] Inoculum consisted of a 70-Klett unit suspension of cells from a 24-h MPSS culture, centrifuged and resuspended in fresh MPSS.

[2] 250-ml Erlenmeyer flasks containing 75 ml MPSS were placed on reciprocating shaker at 82 oscillations/min and shaken for 22 h before inoculation to insure equilibration with atmosphere. Aerobic flasks plugged with cotton; 6% O_2 atmosphere was contained in sidearm flasks sealed with clamp and stopper.

[3] Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values represent averages from duplicate cultures. ND = not determined.

shaking. Investigation of the inconsistency of this response revealed that one important factor was previous exposure of the medium to light. Cotton-stoppered flasks of MPSS medium made in one batch were stored for one week either in a dark cabinet or beside a window. When inoculated in triplicate with 0.5 ml of a 24-h MPSS culture and incubated on a shaker, the medium stored in the dark supported excellent growth in 24 h. No growth occurred in the cotton-stoppered flasks of the medium which had been exposed to sunlight; but sidearm flasks of this medium treated identically did support growth if incubated under 6% O_2.

When an inoculum as small as one loopful of a 24-h culture was incubated in CHSS without agitation, excellent growth appeared consistently after about 96 h. The visible turbidity did not increase gradually during the long incubation period, but appeared rapidly between the third and fourth day. MPSS medium did not support growth from one-loop inocula during this time period; but on one occasion when triplicate flasks of MPSS were kept in the incubator for nine days, growth appeared in all three of them between the seventh and the ninth day. Triplicate flasks of CHSS medium in the same experiment all produced growth by 96 h. All of these cultures were confirmed to be pure by microscopic examination. Whether MPSS would always support growth from a 1-loop inoculum on extended incubation is not known.

Does growth phase of inoculum affect oxygen tolerance? While the size of the inoculum had a direct influence on the ability to initiate growth in an air atmosphere, the age of the culture from which the inoculum was obtained had no effect in CHSS unless the culture was so

old as to be in the death phase. Figures 3 and 4 present data from experiments in which growth curves were compared in flasks of CHSS inoculated with cells taken from cultures in the early and late logarithmic and the stationary phases of growth. Figure 3 is a growth curve of a typical stock culture in 75 ml of MPSS under 6% O_2: 94% N_2 in a sidearm flask incubated without agitation. The inoculum for this culture was 1 ml of the previous serial transfer under similar conditions, subcultured at 24 h (late logarithmic phase of growth). The sidearms of such flasks were not opened during growth of the cultures.

In order to determine whether the condition of the inoculum affected the ability of S. volutans to initiate growth under an atmosphere of air, cells were removed from cultures grown as described above during the early logarithmic (10 h), late logarithmic (24 h), or stationary (31 h) phase of growth. These cells were centrifuged and resuspended in MPSS broth to a turbidity of 70 Klett units, and 1.0 ml of each suspension was used to inoculate 75 ml of CHSS medium in a cotton-stoppered 250-ml Bonner flask which was incubated on a reciprocal shaker (82 oscillations/min). As illustrated in Figure 4, very similar growth curves were obtained for all of these cultures, regardless of the phase of growth of the culture from which the inoculum was obtained. In addition, one-loop inocula from these 70-Klett unit suspensions all initiated growth in cotton-stoppered flasks containing 75 ml of CHSS medium incubated in air without shaking; good turbidity was noted visually between 70 and 80 h of incubation, regardless of the condition of inoculum.

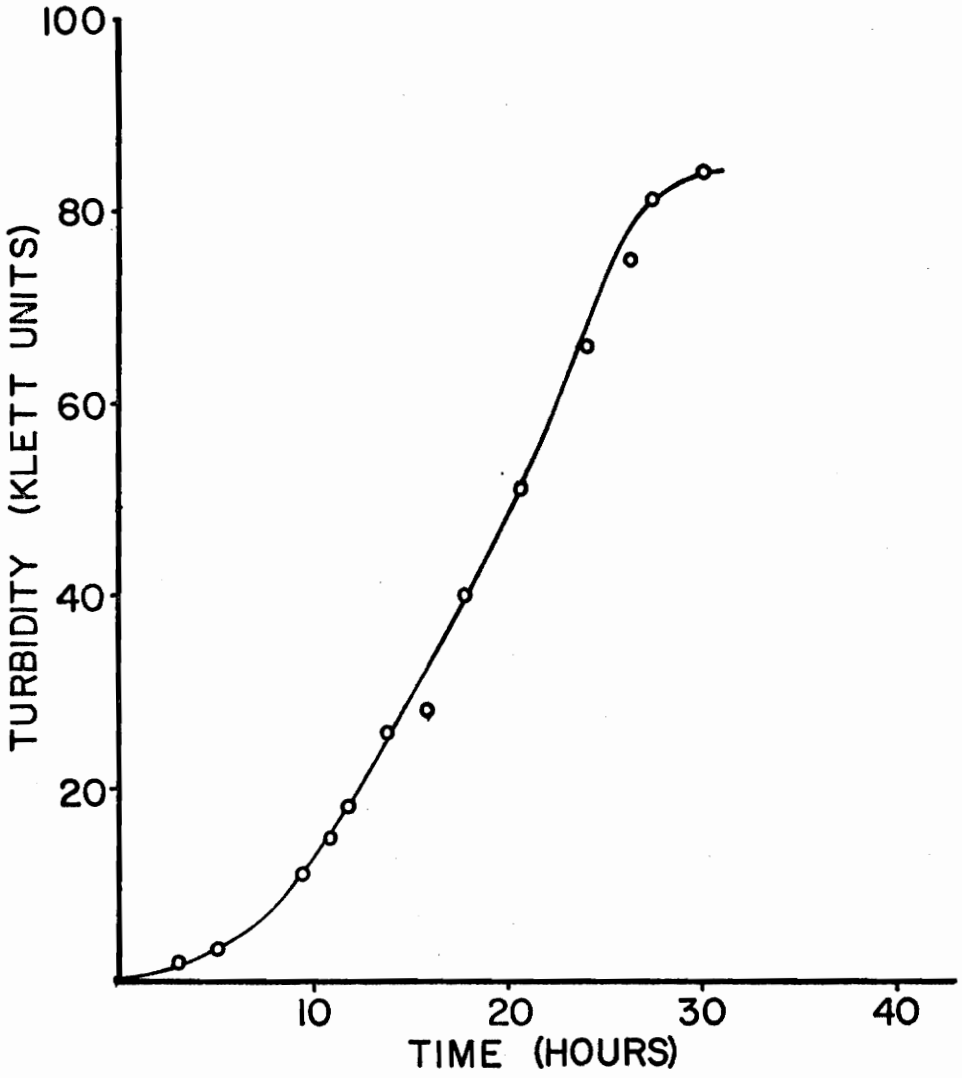


FIGURE 3. GROWTH OF A STOCK CULTURE OF S. VOLUTANS INCUBATED STATICALLY IN MPSS BROTH UNDER 6% O_2:94% N_2. INOCULUM = 1.0 ML OF A SIMILAR 24-H CULTURE.

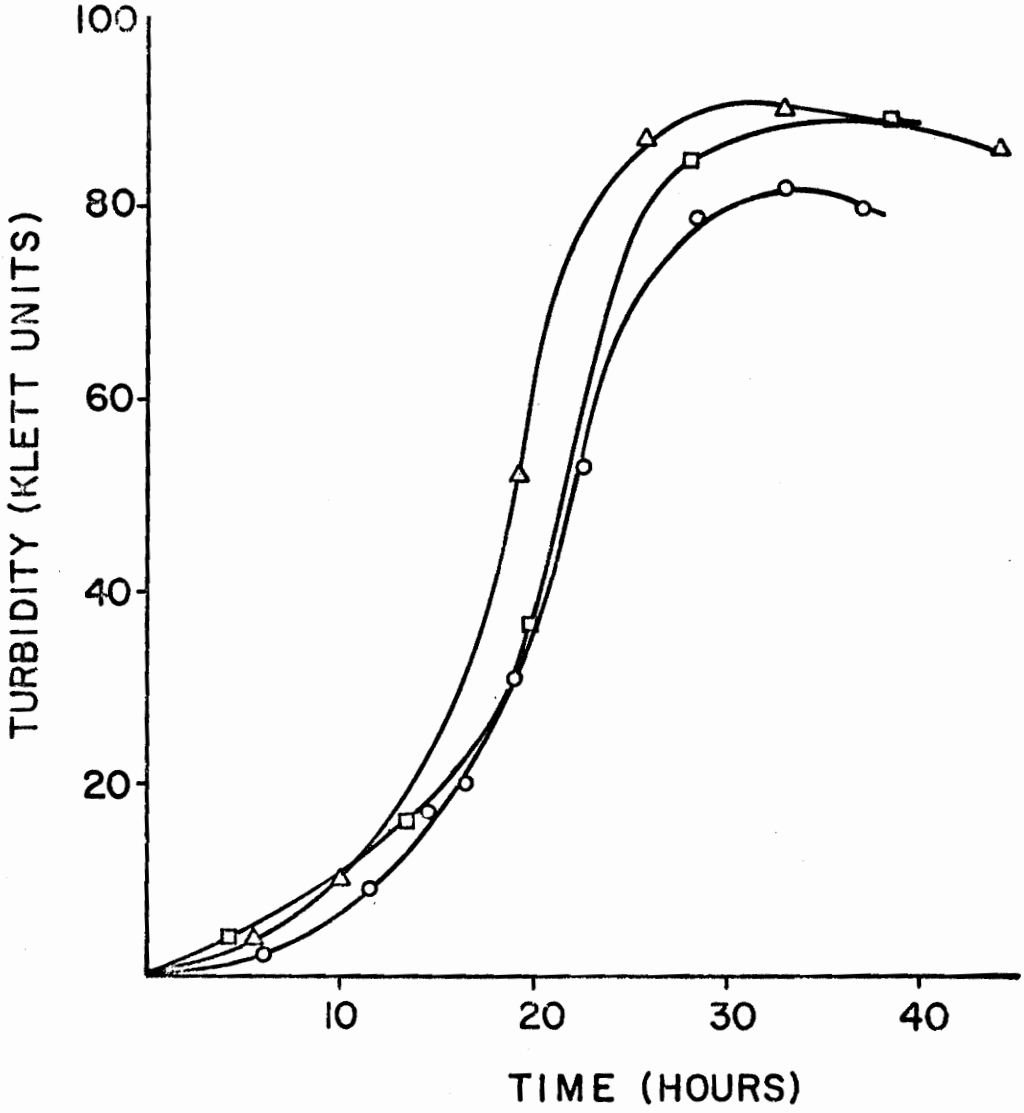


FIGURE 4. EFFECT OF CONDITION OF INOCULUM ON GROWTH OF S. VOLUTANS IN CHSS BROTH WHEN SHAKEN IN AN AIR ATMOSPHERE. o = EARLY LOG PHASE INOCULUM; Δ = LATE LOG PHASE INOCULUM; □ = STATIONARY PHASE INOCULUM.

In Table 16, the results of a different but related experiment are presented. In this case, the inoculum size was not adjusted to be equivalent, but cells were simply removed from the stock culture at various times and inoculated into CHSS medium. Therefore, the data show the combined effects of increasing age of the inoculum culture and increasing inoculum size (older cultures contain greater cell densities). In this experiment, the medium was contained in 50-ml quantities in 250-ml cotton-stoppered Erlenmeyer flasks and was not shaken. The volume of inuculum used was 0.1 ml, and flasks were inoculated from the stock culture when it was aged 11, 24, 36, 48, and 60 h. As shown in Table 16, the turbidity when measured at 60 h shows the long lag period characteristic of growth from a very small inoculum, but the maximum turbidity eventually attained was comparable for inocula from cultures aged 11 to 48 h. In the 60-h culture, many nonmotile cells and fragments were present, and those cells which showed motility were reversing but swam slowly. No growth occurred from an inoculum obtained from this culture. Presumably the number of viable cells was below the inoculum size needed to initiate growth.

Effect of phosphate concentration on oxygen tolerance. The two most unusual physiological characteristics of S. volutans appear to be its obligate microaerophilism and its sensitivity to low concentrations of phosphate in the growth medium. Hylemon et al. (38) have suggested that these two toxicities might be related through the mechanism of the adenylate energy charge and its effect on control of cellular metabolism. In the present work, phosphate was found to be more toxic under an air

TABLE 16

Combined Effects of Size and Condition of Inoculum on Growth of *S. volutans* in CHSS[1]

Age of culture from which inoculum obtained[2] (hours)	Growth response[3] at 60 h	Maximum turbidity[3] attained	Time to attain maximum turbidity (h)
12	8	48	84
24	34	45	72
36	34	34	60
48	38	40	72
60	0	0	NA

[1] 50-ml quantities of CHSS were incubated without agitation in 250-ml cotton-stoppered flasks.

[2] Inoculum = 0.1 ml of stock culture of specified age growing in MPSS; all inocula were obtained from the same stock culture at various times.

[3] Measured in Klett units of turbidity using 16-mm cuvettes and the blue (420 nm) filter.

atmosphere in all three growth media used.

In MPSS medium, growth occurred in 24 h in cotton-stoppered flasks inoculated with 0.5 ml of a 24-h culture and incubated without agitation. Concentrations of added phosphate (K_2HPO_4) of 3 X 10^{-3} M or greater completely inhibited growth under an air atmosphere, but good growth occurred with this concentration of added phosphate under an atmosphere of 6% O_2: 94% N_2.

The concentration of added phosphate needed to inhibit growth in CHSS under an air atmosphere was about 3-fold greater (10^{-2} M) than in MPSS. This toxicity also was relieved by incubation under a 6% O_2 atmosphere, as shown in Table 17.

The fact that S. volutans tolerated both oxygen and added phosphate better in CHSS than in MPSS raised the question of whether peptone contained more phosphate than casein hydrolysate. The phosphate content of 1% solutions of peptone and of casein hydrolysate was determined by the method of Fiske and Subbarow (22).

Four lots of peptone (Difco) were assayed and a considerable range of values obtained. One lot contained 0.067% P; two had 0.085%, and one lot 0.13%. The average of these four values, 0.09%, was used for comparative purposes.

Casein hydrolysate from the lot employed in CHSS medium throughout this work contained 0.051% P. From these figures, the phosphate content of MPSS, which contains 0.5% peptone, is 0.0046 g P/l, or 1.5 X 10^{-4} M phosphate. CHSS, which contains 0.25% casein hydrolysate, thus contains 0.0013 g P/l of medium, or 4.1 X 10^{-5} M phosphate.

TABLE 17

Relationship between Phosphate Toxicity and Atmosphere of Incubation in CHSS[1]

Phosphate added[2] To CHSS	Growth response[3] at 26 h in atmosphere of:	
	Air	6% O_2: 94% N_2
None (control)	87	111
1.5×10^{-2} M	16	95

[1] 250-ml Erlenmeyer flasks containing 75-ml quantities of CHSS were incubated at 30 C on a reciprocating shaker at 82 oscillations/min through a 3.5-cm path. Inoculum = 1.0 ml of a 70-Klett unit suspension in MPSS of cells from a 24-h-old culture in MPSS.

[2] K_2HPO_4 was sterilized by filtration and added to medium aseptically after it had been autoclaved.

[3] Measured in Klett units of turbidity using 16-mm cuvettes and the blue (420 nm) filter. Values presented are averaged from duplicate cultures.

According to these data, although there is almost 4-fold more phosphate in MPSS than in CHSS, even the higher level is far below the range in which phosphate becomes inhibitory, as shown by the comparison in Table 18. Thus the organism apparently is tolerant of approximately 3-fold higher total phosphate levels (inherent + added) in CHSS than in MPSS.

The toxic effects of phosphate appeared to be exerted on growth initiation and not on growth already in progress. If an inhibitory concentration of phosphate was present when the medium was inoculated, growth was inhibited; but if the same concentration of phosphate was added after the culture was in the logarithmic phase of growth, no inhibition occurred. The results of a typical experiment are shown in Figure 5.

In DGM, the concentration of phosphate routinely used was 10^{-4} M. If this amount was increased to 10^{-2} M, growth was inhibited; and again this inhibition was reversed by incubating the culture under 6% oxygen, as shown in Table 19.

It was possible that the increased phosphate might be interfering with iron availability, perhaps by precipitating with ferric ions. However, although this possibility has not been ruled out, a 6% O_2 incubation atmosphere relieved the toxicity of phosphate even though the medium had been stored and the phosphate added under atmospheric air. In addition, a tenfold increase in the concentration of $FeCl_3$ (to 30 mg/l) did not relieve the toxicity of phosphate (Table 19).

A series of experiments was conducted using a standard small

TABLE 18

Comparison of Phosphate Levels in MPSS and in CHSS, Showing that Difference in Sensitivity to Added Phosphate Does Not Result from Inherent Difference in Phosphate Concentration

Medium	Phosphate inherent in medium	Inhibitory concentration of added phosphate	Total phosphate (inherent + added)
MPSS	1.5×10^{-4} M	3×10^{-3} M	3.15×10^{-3} M
CHSS	4.1×10^{-5} M	1×10^{-2} M	10.04×10^{-3} M

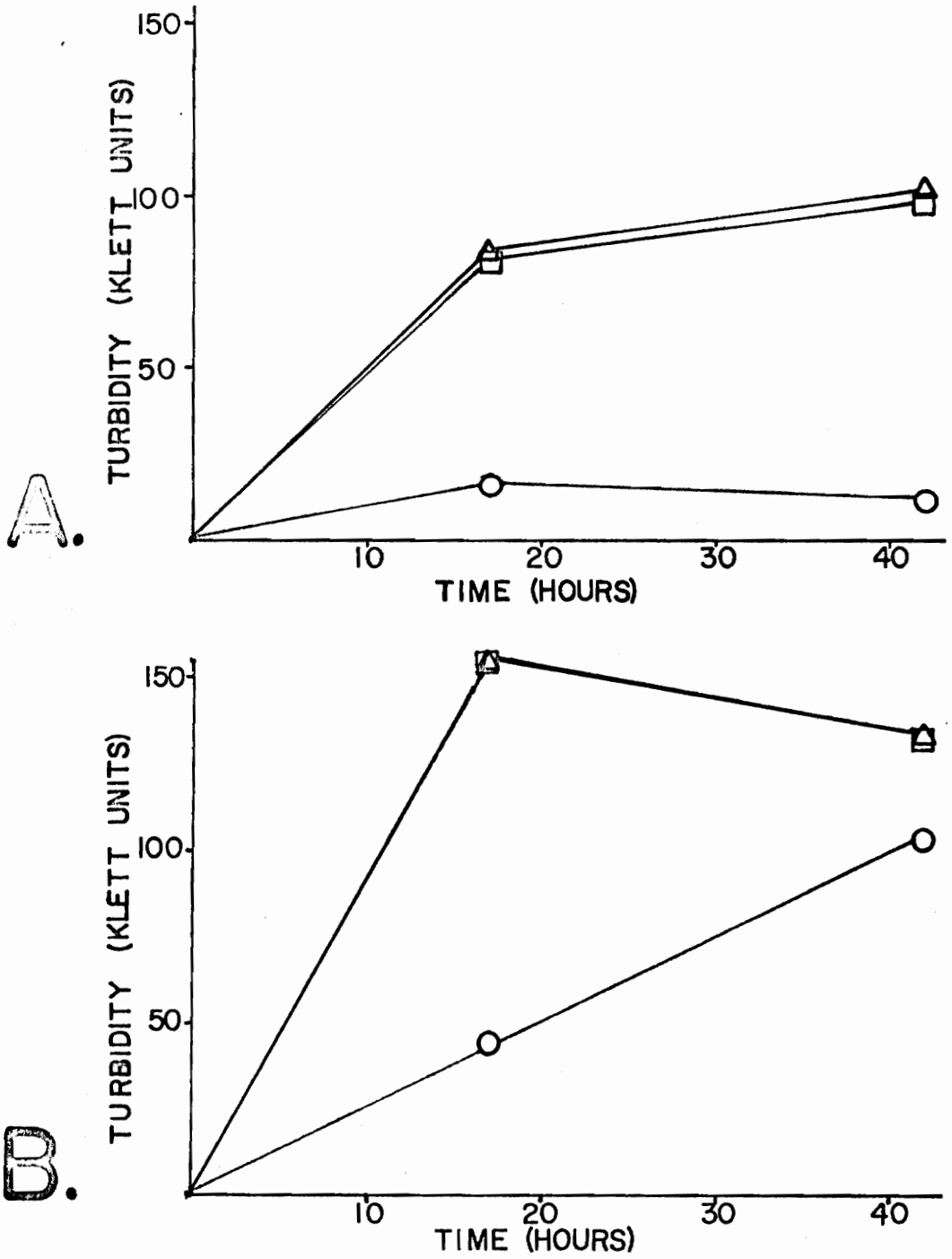


FIGURE 5. EFFECTS OF 10^{-2} M PHOSPHATE, ADDED AT 0 h OR AT 17 h, ON GROWTH OF S. VOLUTANS IN CHSS BROTH. (30 ml medium inoculated with 0.5 ml of a 100-Klett-unit suspension in DMM of cells from a 24-h MPSS culture; incubated with shaking.) A = AIR ATMOSPHERE; B = 6% O_2. O = +PO_4 at 0 h; Δ = +PO_4 at 17 h; □ = CONTROL; NO PO_4 ADDED.

TABLE 19

Relationship between Phosphate Toxicity to S. volutans and Atmosphere of Incubation in Defined Growth Medium[1]

Phosphate concentration	Growth response[2] in atmosphere of:	
	Air	6% O_2: 94% N_2
10^{-4} M	30	25
10^{-2} M	6	26

[1]Medium = DBM with 30 mg/l $FeCl_3 \cdot 6H_2O$ and 10^{-6} M L-epinephrine, contained in 5-ml quantities in cotton-plugged tubes. Inoculum = 1 drop of a culture grown for 24 h in DGM.

[2]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter. Values presented are averaged from duplicate tubes.

inoculum in DBM in an attempt to clarify the relationship among the various factors affecting growth initiation. From Table 20, it can be seen that growth under air from this small inoculum was prevented either by omission of the iron chelator or by an increase in the phosphate content of the medium to 10^{-2} M. Both of these inhibitions could be reversed by incubation under an atmosphere of 6% O_2: 94% N_2.

Effects of Iron in the Cultivation of Campylobacter fetus

Because increased levels of iron or addition of exogenous iron chelators lessened the toxicity of oxygen for S. volutans, it was of interest to know whether another well-known obligate microaerophile, Campylobacter fetus, would be similarly affected.

In a preliminary experiment with C. fetus subsp. jejuni, strain H840, growth from a 1-loop inoculum occurred in 5 ml of Albimi Brucella Broth after four days of incubation in a candle jar but not in air. The same medium with 10^{-5} M L-arterenol added supported visible growth within two days in the candle jar, and heavy growth occurred in an air atmosphere by the fourth day. Growth in a candle jar /which contains an atmosphere of ca. 17% O_2 and 2.5% CO_2 (49)/, but not in air, occurred on plates of Albimi agar supplemented with 30 mg/l $FeSO_4 \cdot 7H_2O$ or with 10^{-5} M arterenol. No growth occurred on unsupplemented plates even in the candle jar.

The stimulatory effect of iron on C. fetus subsp. jejuni, strain H840, was further studied in a series of streak plate experiments on Albimi agar with various added compounds.

Figure 6 shows the effects of $FeSO_4 \cdot 7H_2O$ (sterilized by filtration

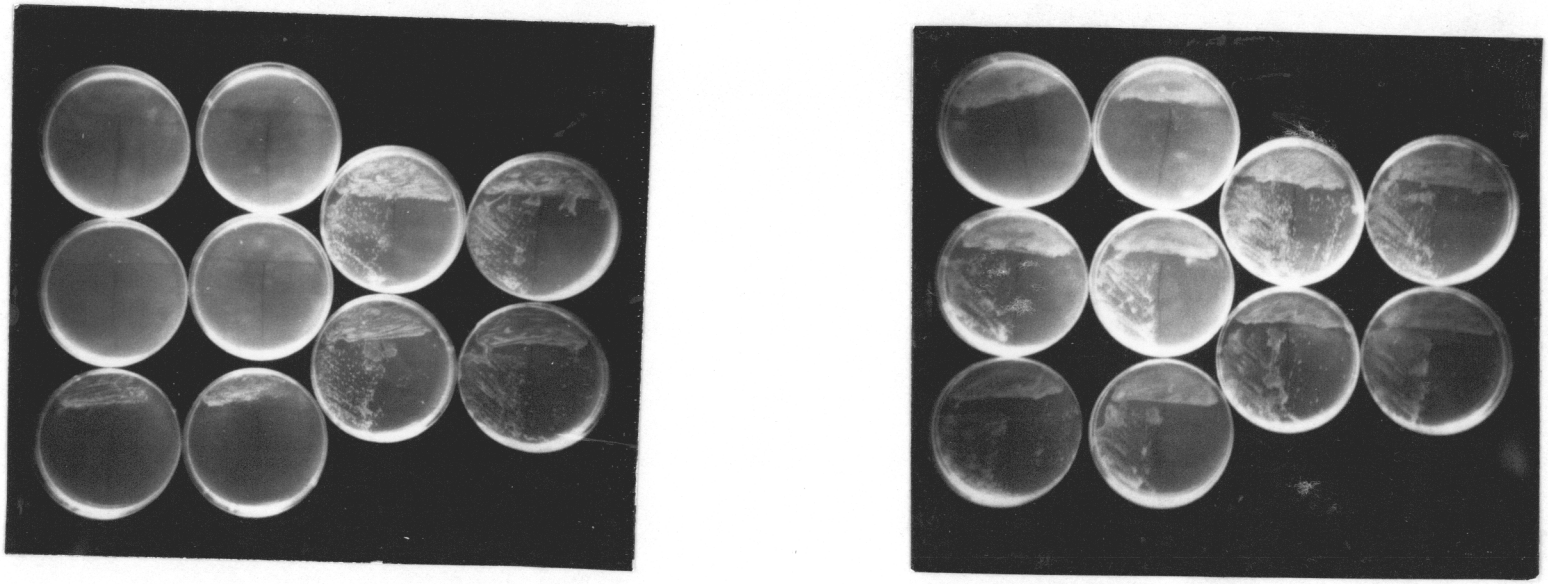
TABLE 20

Effects of L-Arterenol and of Phosphate on Growth of S. volutans in DBM under an Atmosphere of Air or of 6% O_2: 94% N_2

Medium[1]	Growth response[2] at 84 h under atmosphere of	
	air	6% O_2: 94% N_2
DBM + 10^{-5} M L-arterenol	73	86
DBM, no iron chelator	0	78
DBM with 10^{-2} M PO_4 + 10^{-5} M L-arterenol	0	89

[1]DBM contains 10^{-4} M PO_4. Medium was contained in 5-ml quantities in 20 X 125 mm culture tubes with loosened screw caps. Inoculum = 2.1 X 10^4 cells/ml medium; cells harvested from 24-h culture grown in MPSS; cells washed 2X in DMM and suspended to a turbidity standard.

[2]Measured in Klett units of turbidity, using 16-mm cuvettes and the blue (420 nm) filter; values averaged from duplicates.



A B

FIGURE 6. EFFECT OF FERROUS SULFATE ON GROWTH OF C. FETUS ON ALBIMI AGAR. A: INCUBATED IN CANDLE JAR; B: INCUBATED IN SEALED JAR, −400 mm Hg AIR, +20 mm Hg CO_2. $FeSO_4 \cdot 7H_2O$ ADDED TO DUPLICATE PLATES AS FOLLOWS:

UPPER LEFT: NONE
MIDDLE LEFT: 0.005%
LOWER LEFT: 0.01%
UPPER RIGHT: 0.02%
LOWER RIGHT: 0.05%

and added aseptically to sterile Albimi agar) at concentrations ranging from 0.005% to 0.05% under the atmospheric conditions of (1) a candle jar and (2) a desiccator in which the air pressure was decreased from 712 to 312 mm Hg, and 20 mm Hg of CO_2 was added. (Blacksburg is located at an altitude of 2200 feet above sea level.) As shown by the photographs, growth occurred under the latter atmosphere in unsupplemented medium but was enhanced by added iron. In the candle jar, however, addition of at least 0.01% ferrous sulfate was required for growth to occur at all, and higher levels were needed to produce growth beyond the first quadrant of the plate.

From Figure 7 it can be seen that added atmospheric CO_2 also was necessary for growth of C. fetus unless the oxygen level was lowered substantially. Even in the presence of 20 mm Hg of added CO_2, the organism did not grow well under 17% O_2 unless ferrous sulfate was added. Addition of 0.05% sodium bisulfite produced slight stimulation in the absence of added CO_2. The stimulatory effect of 0.05% added bisulfite is much more striking when dilution plates are used rather than streak plates (N. R. Krieg, personal communication). Figure 7 also shows that the needs for added iron and for CO_2 were obviated by lowering the level of atmospheric oxygen to approximately 9% (- 400 mm Hg air pressure). These plates showed excellent growth on Albimi agar with no supplements of any kind.

Figure 8 shows that ferric iron appeared to be as effective as the ferrous form in promoting growth of C. fetus. Solutions of ferrous sulfate were sterilized by filtration and added to the medium at final

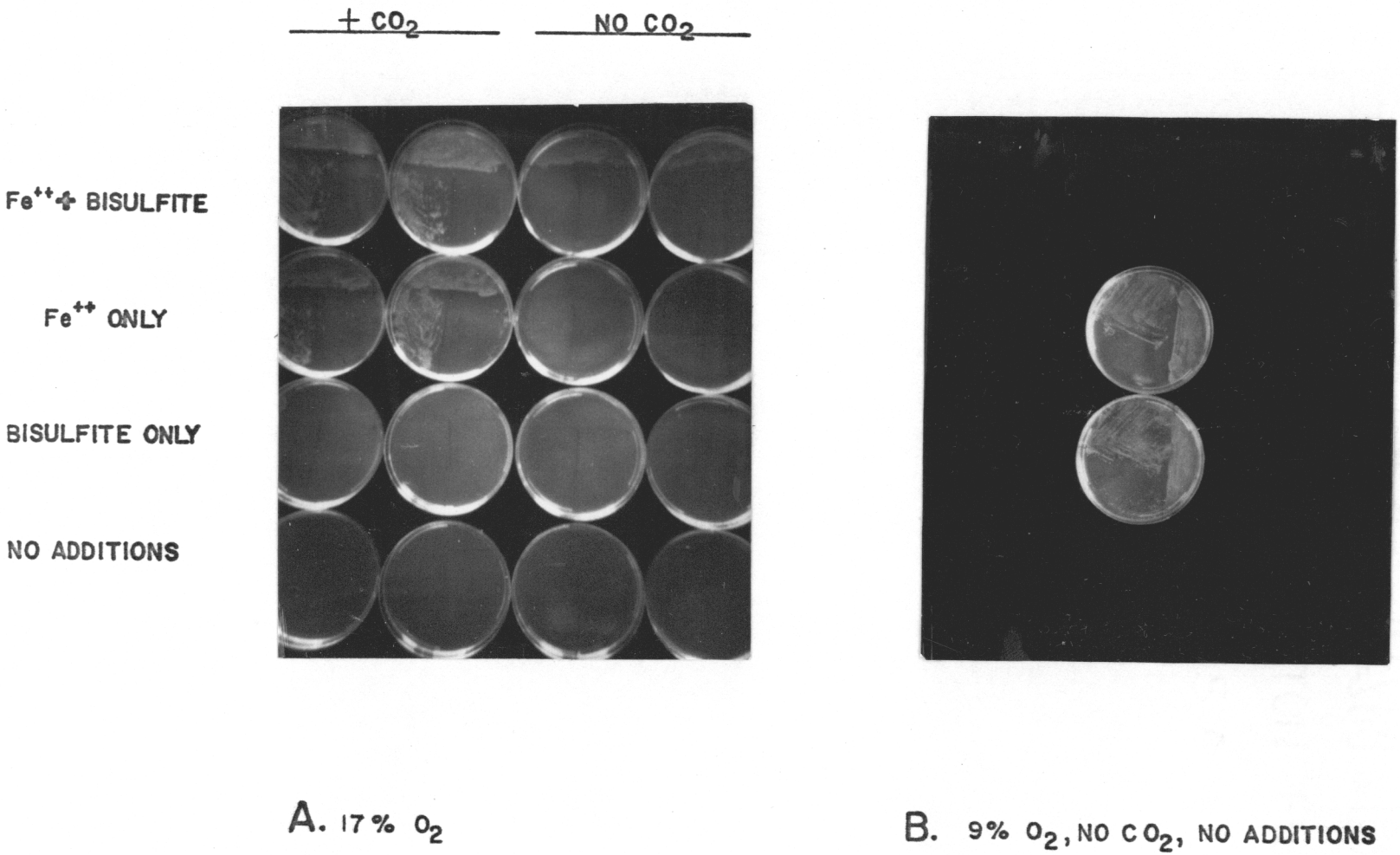
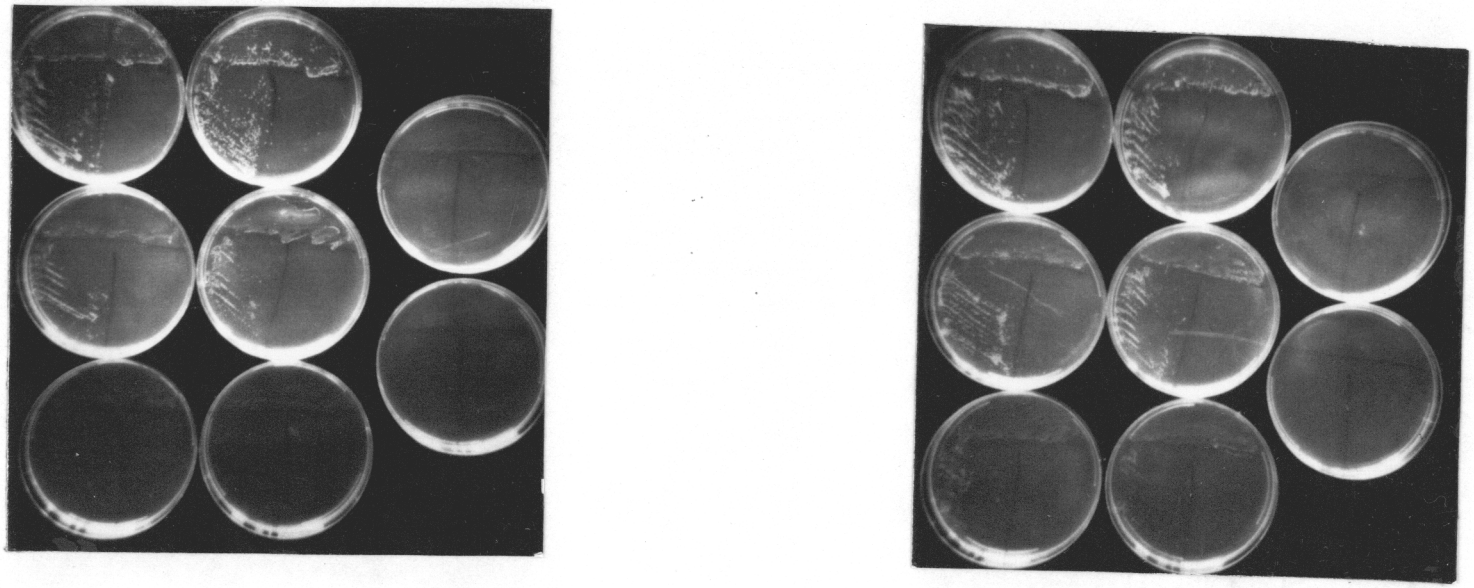


FIGURE 7. EFFECTS OF CO_2, FERROUS SULFATE, AND SODIUM BISULFITE ON GROWTH OF C. FETUS ON ALBIMI AGAR. AIR REMOVED FROM JAR TO $\%O_2$ INDICATED. "+ CO_2" = 20 mm Hg CO_2 ADDED. ADDITIONS TO MEDIUM: "Fe^{++}" = 0.05% $FeSO_4 \cdot 7H_2O$. "BISULFITE" = 0.05% SODIUM BISULFITE.



A. B.

FIGURE 8. EFFECTS OF FERROUS AND OF FERRIC IRON ON GROWTH OF C. FETUS.
ATMOSPHERE: AIR; 70 mm Hg ADDITIONAL AIR AND 20 mm Hg CO_2 ADDED.
MEDIUM: ALBIMI AGAR + 0.6% BES + 0.05% SODIUM BISULFITE + 0.1% $NaHCO_3$.
A. $FeSO_4 \cdot 7H_2O$ ADDED IN FOLLOWING AMOUNTS: UPPER LEFT PAIR OF PLATES, 0.05%
MIDDLE LEFT PAIR, 0.02% RIGHT PAIR, 0.005%
LOWER LEFT PAIR, 0.01%
B. EQUIVALENT AMOUNTS OF IRON ADDED AS FERRIC SULFATE.

concentrations ranging from 0.005% to 0.05% after the medium had been autoclaved. Ferric sulfate was added in the same manner to the other series of plates, in quantities which provided equivalent amounts of iron.

This experiment also demonstrated that, when supplementary iron and CO_2 were supplied, the organism grew well even in an atmosphere containing 21% oxygen. These plates were incubated in a sealed jar to which 70 mm Hg of additional air was added under positive pressure to compensate for the effect of altitude in lowering the atmospheric pressure below 760 mm Hg. An additional 20 mm Hg of CO_2 also was added. 0.05% sodium bisulfite was added to all plates. The medium also contained 0.1% $NaHCO_3$, and BES buffer at 0.6% was added to prevent acidification of the medium by the added CO_2. It has since been shown (N. R. Krieg, personal communication) that the bicarbonate and BES are not necessary for growth and do not affect the results.

In Figure 9, the results are presented from an experiment in which arterenol or DOPA was substituted for added iron in plates of Albimi agar. Excellent growth was obtained on medium containing these compounds at a concentration of 10^{-4} M. Less growth occurred when the concentration was lowered to 10^{-5} M, and no growth occurred on unsupplemented plates. These plates again were incubated in a sealed jar to which 70 mm Hg of air and 20 mm Hg of CO_2 were added to simulate air pressure at sea level with the full amount of atmospheric oxygen but enriched in CO_2. The Albimi agar in these plates contained additions of 0.05% sodium bisulfite, 0.6% BES, 10 mg% biotin, and

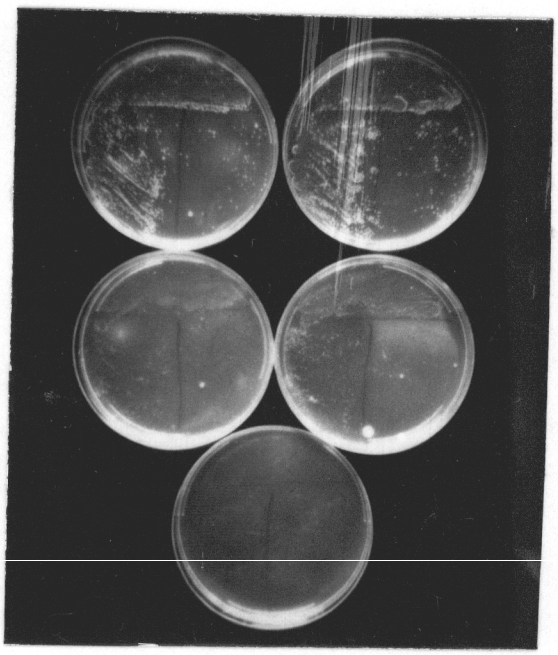


FIGURE 9. EFFECTS OF DOPA AND ARTERENOL ON GROWTH OF C. FETUS ON ALBIMI AGAR WITHOUT ADDED IRON.
ATMOSPHERE: AIR + 70 mm Hg ADDITIONAL AIR + 20 mm Hg CO_2.
LEFT PLATES: TOP, 10^{-4} M DOPA; MIDDLE, 10^{-5} M DOPA.
RIGHT PLATES: TOP, 10^{-4} M ARTERENOL; MIDDLE, 10^{-5} M ARTERENOL.
BOTTOM PLATE: NO DIHYDROXYPHENYL COMPOUNDS ADDED.

0.1% $NaHCO_3$. Further work (N. R. Krieg, personal communication) has shown that the BES, bicarbonate, and biotin are not necessary and do not affect the results.

In the experiment illustrated in Figure 10, inocula were derived from cultures in semisolid Albimi agar with and without 0.05% added ferrous sulfate. These inocula were plated on Albimi agar with and without added ferrous sulfate and incubated in the "sea level air" atmosphere with added CO_2. The plates contained 0.6% BES and 0.05% sodium bisulfite in Albimi agar. In subsequent experiments (N. R. Krieg, personal communication), the same results have been obtained in the absence of BES. From this experiment it can be seen that prior growth in the presence of added iron did not eliminate the requirement for added iron when the cultures were incubated in an air atmosphere with added CO_2.

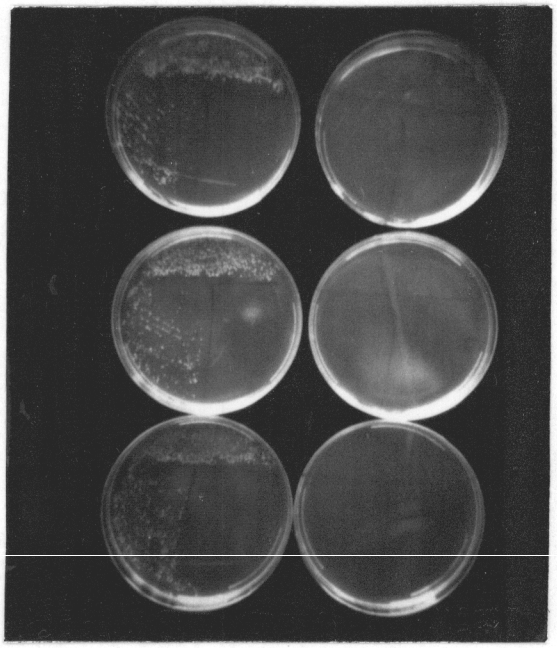


FIGURE 10. REQUIREMENT FOR ADDED IRON FOR AEROBIC GROWTH OF C. FETUS ON ALBIMI AGAR REGARDLESS OF PREVIOUS GROWTH IN IRON-SUPPLEMENTED MEDIUM.
ATMOSPHERE: AIR + 70 mm Hg ADDITIONAL AIR + 20 mm Hg CO_2.
LEFT PLATES: 0.05% $FeSO_4 \cdot 7H_2O$ ADDED TO MEDIUM.
RIGHT PLATES: NO ADDED IRON.

INOCULA DERIVED FROM CULTURES GROWN IN SEMISOLID ALBIMI MEDIUM AS FOLLOWS:
TOP: 48 H, NO ADDED IRON.
MIDDLE: 24 H, NO ADDED IRON.
BOTTOM: 24 H, 0.05% $FeSO_4 \cdot 7H_2O$ ADDED.

DISCUSSION

Part 1: Spirillum volutans as a Monitor for Industrial Pollutants

Monitoring of water quality with respect to industrial pollutants, both in streams and in effluents about to be discharged into streams, is an essential component of the multiple-use management of water resources (12). Biological monitoring has received considerable attention as a useful supplement to (but not a substitute for) monitoring by chemical or physical methods (11). Biological monitors will not identify the pollutants in a complex effluent, but they could indicate the occurrence of adverse biological effects which might be difficult to predict by analyzing physical and chemical parameters alone. Because there is no one "representative" aquatic organism which can serve as an index of what is harmful to the entire aquatic community, it is desirable to have different systems available which could utilize a variety of species.

Cairns *et al.* (12) have emphasized the need for bioindicators capable of exhibiting rapid responses to industrial pollutants. While *S. volutans* is not an "economically important" organism, it offers several advantages for use in a biological monitoring system. The response to uncoordinating agents is rapid and unmistakable. Because *S. volutans* is readily cultivable, large numbers of organisms of comparable physiological state could be kept readily available. Problems which may be encountered when using higher organisms, such as aging, physiological effects of confinement, and adaptation through long-term, low-level exposure to toxicants, are largely avoided by using a bacterial system.

The exceptionally large size of this bacterium makes observation of the motility, and even of the fascicles of flagella, feasible even at low magnifications using darkfield microscopy.

Because the effects of various toxicants vary widely among different species of aquatic life (53), no one organism can be used to predict the effect of pollutants on all other species, or on the aquatic ecosystem as a whole. Therefore, the use of a biological monitoring system would involve detecting the presence of toxicants should they rise to levels previously defined as harmful if discharged into the stream. These levels could be determined by actual study of the particular receiving stream, including such factors as its assimilative capacity, the presence of toxicants from other industrial sources, and the effects of the agents in question on its biotic community. Biological harm to a stream would, of course, have to be assessed not only in terms of dramatic effects such as fish kills, but in terms of the ability of the members of the ecosystem to function adequately throughout their complete life cycles.

Use of the Spirillum volutans system for effluent monitoring would involve determination of the sensitivity of the cells to the potential pollutants in question in the presence of the other components of the effluent. A suitable dilution of effluent then could be made which would just permit normal motility if the pollutants were at the highest level considered permissible for discharge. Upstream water could be used as the diluent to simulate the interaction of the effluent with the receiving stream water. Tests on incoming stream water would distinguish

pollution originating from spills within the plant from toxicants already present upstream.

With these techniques, uncoordination of the spirilla by effluent would indicate a rise in one or more toxic substances to an unacceptable level. Tests of effluent from various portions of the industrial process could indicate the source of the spill within the plant. The waste could then be subjected to physical or chemical tests to determine the identity and concentration of the particular toxicants involved. Meanwhile, the waste could be shunted to a holding pond for further treatment if necessary.

In studies reported by Cairns et al. (11), in which bluegill movement patterns and breathing rates were tested for use in an industrial effluent monitoring system, zinc was employed as the toxicant and was detected at concentrations of a few parts per million. A latency period of several hours elapsed between the introduction of the zinc and the detection of the response, and the length of the latency period was inversely proportional to the concentration of the toxicant employed. The lowest concentration of zinc detected was 2.55 ppm. Thus, it appears that the maximum sensitivities of the two biological monitors, using bluegills and using S. volutans, are comparable for zinc. The extent of other similarities or differences in sensitivity of the two systems is not known at present, but it may be possible to use different biological monitors to reinforce or complement each other.

Toxicants which are bioaccumulated and slow in their action, such as DDT and polychlorinated biphenyls, would be unlikely to be detected

by any biological monitor which depends on short-term responses. However, growth effects of polychlorinated biphenyls over a time span of several days have been demonstrated in cultures of Chlamydomonas (50).

Another problem which may be encountered when using any biological monitor is that turbid effluents may contain toxicants present as precipitates or adsorbed to particles. In such cases, pretreatment of the sample (for example, by solubilization with acid and readjustment of pH) may be necessary in order to perform a valid test.

Part 2: Nutrition and Oxygen Sensitivity of S. volutans and C. fetus

Oxygen is toxic in some degree to all organisms known (34). It would seem, however, that an organism with both a strictly oxidative metabolism and a degree of oxygen sensitivity much greater than that of most aerobes would face a particularly difficult set of circumstances. Two notable features of the metabolism of S. volutans are its oxygen sensitivity and its sensitivity to small amounts of phosphate (37), which inhibits its respiration (19) as well as its growth. The fact that growth of S. volutans is inhibited by both phosphate and oxygen suggested the possibility (38) that the two phenomena might be related, and that phosphate sensitivity might provide an explanation for the obligate microaerophilism of the organism.

Atkinson (1) has described the function of the adenylate energy charge in the regulation of metabolism. The adenylate charge is a measure of the "energy level" of the cell and is defined as

$$\frac{[\text{adenosine triphosphate}] + \frac{1}{2}[\text{adenosine diphosphate}]}{[\text{adenosine triphosphate}] + [\text{adenosine diphosphate}] + [\text{adenosine monophosphate}]}.$$

The charge for a wide variety of living cells is strongly stabilized at a value of about 0.8 (17). Regulatory enzymes in pathways which generate ATP decrease in activity at high levels of energy charge, and enzymes regulating ATP-consuming reaction sequences become more active as the charge increases.

One hypothesis concerning the microaerophilism of S. volutans is that a defect exists in the control of energy generation by adenylate

charge. According to this hypothesis, in the presence of ample inorganic phosphate and oxygen, respiration would proceed at an unregulated rate until the adenylate charge greatly exceeded the value characteristic of most regulated cells. The excess of ATP might then "shut off" enzymes not defective in their susceptibility to control and also allow biosynthetic reactions to proceed unregulated by the availability of ATP. A cell thus defective in internal regulation of its energy-generating processes might require external regulation in the form of limitation of the supply of oxygen, inorganic phosphate, or both.

Koobs (41) has discussed the regulation of the relative rates of glycolysis and respiration in mammalian cells by competition for a limited amount of inorganic phosphate. While S. volutans does not possess a glycolytic pathway, this paper nevertheless lends support to the idea of respiratory regulation by phosphate availability.

The hypothesis of increased and unregulated ATP production in the presence of excessive concentrations of PO_4 or or O_2 would seem to predict at least a temporary increase in respiratory rate under these conditions. No information is available concerning the relative rates of respiration of S. volutans under atmospheres of air and of 6% O_2: 94% N_2. The low value reported by Cole and Rittenberg (19) for oxygen consumption by S. volutans under an air atmosphere (30 nmoles/mg protein/min, measured in 50 mM phosphate buffer with 5 mM succinate as substrate) was in contrast with values of 60 for Aquaspirillum itersonii and 127 for A. serpens, measured under the same conditions. Although the units of measurement and experimental conditions are not directly comparable,

the Q_{O_2} value reported by Caraway and Krieg (14) for S. volutans in DMM with 10^{-2} M succinate would seem to indicate a rather high respiratory rate.

It must also be considered that Cole and Rittenberg (19) reported that 5 mM phosphate inhibited the respiratory rate of S. volutans, but not of the other two spirilla, by 30-40%. The hypothesis discussed above would predict an increase in respiration (or at least in ATP generation) with added phosphate. Cole and Rittenberg measured oxygen consumption polarographically and reported it as a rate per minute, but they did not specify how soon measurement was begun after adding phosphate to the cell suspension. It would be of interest to record the response of a suspension of S. volutans, already respiring at a given rate, to a sudden addition of phosphate to the medium. Possibly an initial stimulation of respiration would occur, representing uncontrolled oxidative phosphorylation, which would later give way to inhibition. Perhaps relevant to this point is the finding by Harrison and Maitra (reviewed in reference 33) that the turnover time of ATP in growing cells is of the order of a few seconds; the data of Chapman, Fall, and Atkinson (17) show large changes in energy charge occurring on a time scale of a few minutes.

A related possible explanation for oxygen sensitivity in S. volutans is the finding with several organisms (33) of increased oxygen consumption with decreased growth efficiency at dissolved oxygen levels below a critical value. This phenomenon was interpreted by Harrison as an uncoupling of oxidative phosphorylation at low values of oxygen tension.

If the coupled respiration of S. volutans were unregulated, perhaps uncoupling of respiration at low oxygen concentrations would lower the energy charge to an acceptable value.

The work of Chance (15) suggested a possible mechanism for an increase in energy charge in oxygen poisoning. The pathway of reverse electron flow consumes ATP and also drains electrons from the succinic dehydrogenase reaction which otherwise would enter the respiratory chain. Chance reported that this pathway was the initial site of inhibition by oxygen and that the effects of this inhibition included a large rise in the ratio of ATP to ADP + AMP. In this case, reverse electron flow acts to lower the energy charge, and inhibition of the pathway allows hypernormal amounts of ATP to be formed. If this pathway in S. volutans were defective or exceptionally susceptible to inhibition by oxygen, increased sensitivity to both phosphate and oxygen might result.

The demonstration that increased phosphate did in fact increase the toxicity of oxygen to S. volutans growing in MPSS or in CHSS lent support to the hypothesis of a relationship, perhaps causal, between the two toxicities. The organism, however, tolerated three times as much added phosphate in CHSS as in MPSS, and this difference could not be accounted for by the small difference between the initial concentrations of phosphate inherent in the two media. For these reasons, it was highly desirable to develop a defined growth medium in which the effects of known concentrations of phosphate on oxygen sensitivity could be studied under known and reproducible nutritional conditions.

Development of a defined growth medium, however, proved to be a complicated problem in itself and eventually revealed another nutritional requirement of S. volutans which could be relieved by low oxygen tension.

The occurrence of growth in a medium containing only "vitamin-free, salt-free" casein hydrolysate, succinate, and mineral salts would seem to suggest the probable absence of extensive requirements for vitamins and other growth factors; yet attempts to substitute "synthetic" casein hydrolysate proved futile. The discovery that the growth factor in casein hydrolysate could be replaced by autoclaved tyrosine replaced one enigma with another until the discovery of Hutner's commentary (36) on the work of Gorini and Lord (25). Actually several investigators have come upon requirements for iron chelators by this route. For example, Neilands (reviewed in 43) reported that Micrococcus leisodeikticus grew only if glucose was autoclaved in the defined medium, and in Lankford's work (43) with B. subtilis var. niger, lag time was reduced by an autoclaved solution of glucose and potassium phosphate ("GP factor").

While iron uptake by S. volutans was not measured directly (a process fraught with experimental pitfalls [43]) and correlated with the ability to grow in DGM, several results supported the interpretation that the function of dihydroxyphenyl compounds is to facilitate iron uptake. Growth was supported by high concentrations of ferrous iron. Growth with ferric iron was effectively supported by a number of different o-dihydroxyphenyl compounds. Both the D and the L isomers of

these compounds appeared to be equally effective at concentrations as low as 10^{-6} M. These results suggested that the dihydrolyphenyls serve a common function rather than that a specific metabolic intermediate is required. Weight is added to this evidence by the extensive similarities between S. volutans and other organisms which have been reported in the literature to require chelators for ferric iron.

Elucidation of the requirement of S. volutans for ferrous iron or for iron chelators provided plausible explanations for some other observations which had been made. The formation of abnormally long, filamentous cells under conditions of iron deficiency has been reported for Mycobacterium smegmatis, Clostridium perfringens, Escherichia coli, and Corynebacterium diphtheriae (43). In the case of M. smegmatis, this defect was related to curtailment of DNA synthesis. In E. coli, iron was shown to be essential for the activity of ribonucleotide reductase. These observations may explain the elongated cells of S. volutans which were present in early and inadequate formulations of the defined medium.

The long lag periods noted in media without iron chelators, and the erratic growth responses obtained with small inocula, closely resemble the observations by Lankford and coworkers (43) concerning the inoculum-dependent lag of Bacillus megaterium. In all media used with S. volutans during the present work, small inocula grew only after a long lag period, if at all, when incubated under an air atmosphere. A striking example noted early was the sudden appearance of heavy growth on the fourth day in flasks of CHSS which had been inoculated with one

loopful of culture; turbidity did not increase gradually during the incubation period. In the case of B. megaterium, Lankford (43) demonstrated that the inoculum-dependent lag represented the time necessary for synthesis of the iron chelator schizokinen to the critical concentration for initiation of growth. For a given inoculum size, the lag period could be shortened or eliminated by adding graded amounts of schizokinen or culture filtrate to the medium.

Similar observations were made with Francisella tularensis (31, 32), which initiated growth from small inocula only in the presence of filtrate from its own cultures and otherwise required an inoculum of 10^5 to 10^8 cells. Both B. megaterium and F. tularensis were shown to begin exponential production of their iron chelators upon inoculation and to continue production at this rate throughout the lag phase of growth. Lankford (43) cited several examples of organisms which were capable of synthesizing chelators for ferric iron and thus were not, strictly speaking, "siderochrome auxotrophs," but which were stimulated by the addition of exogenous iron chelators or of culture filtrate. As in the case of S. volutans, Lankford reported that "unnatural" chelators used with B. subtilis var. niger, including o-dihydroxyphenyl compounds, exhibited an optimal concentration and became inhibitory when present at higher concentrations.

The fact that large inocula of S. volutans were able to initiate growth in the absence of added iron chelators suggested that the organism is capable of synthesizing an iron chelating compound which promotes its own growth. The preliminary experiments showing growth from

smaller inocula in the presence of culture filtrate from S. volutans supported this possibility. However, the cultural conditions used to obtain the filtrate (DBM containing 30 mg/l $FeSO_4 \cdot 7H_2O$) probably did not favor maximal synthesis of an endogenous chelator for ferric iron. Lankford (43) reported that maximal synthesis of such compounds usually is obtained when the concentration of iron in the medium is barely sufficient to support maximal growth. Filtrates from cultures of S. volutans grown from a relatively large inoculum in DBM containing only a small amount of ferric iron, and no exogenous chelators, might be highly stimulatory to the growth of small inocula under the same conditions. Filtrates from cultures of aerobic spirilla (genus Aquaspirillum) might also provide a source of iron chelators for S. volutans. The growth of this organism in mixed cultures and dialysis-sac cultures in the early experiments of Rittenberg and Rittenberg (55) may as easily be attributable to chelation of iron by the other bacteria as to reduction of oxygen tension. It would be of interest to obtain growth curves using small inocula and various amounts of culture filtrate. Such data, rather than measurements of final turbidity only, would distinguish a reduction of the lag phase from other possible stimulatory effects, such as increases in growth rate or final yield.

One characteristic of S. volutans, not mentioned in connection with any of the other organisms discussed above, was that the long lag phase of small inocula and the need for iron chelators were eliminated by incubation under microaerophilic conditions. The reason for this relationship is not yet clear. Gottlieb (26) reported that exposure to

oxygen at 1 to 4 atmospheres prolonged the lag phase of growth of mycobacteria, Achromobacter, Pseudomonas aeruginosa, Enterobacter aerogenes, and E. coli. Prolongation of lag also was noted in L-strain fibroblasts exposed to an atmosphere of more than 5% O_2 (34) and in Clostridium perfringens when the E_h of the culture medium was raised (26). It would be of interest to determine whether supplementation with large amounts of iron or with iron chelators might reverse this effect of increased O_2. Perhaps a more general relationship exists between iron and O_2 toxicity, with varying degrees of sensitivity among organisms.

It is possible that lowered oxygen tension in cultures of S. volutans allows some of the iron to exist in the ferrous state and thus to be available to the organism. However, in media prepared and stored under aerobic conditions, and containing iron added in the ferric state, growth was initiated promptly from small inocula in cultures placed in the microaerophilic atmosphere after inoculation. Rapid reduction of ferric iron in the medium would be required in order to explain the initiation of growth under these circumstances, and this explanation seems rather unlikely.

There are a number of other possible links between iron metabolism and oxygen toxicity which should be considered. Catalase activity, known to be very weak in S. volutans (19, 38), is lowered in states of iron deficiency in bacteria (43). Iron deficiency may either explain the weak catalase activity of S. volutans or further aggravate it, contributing to the oxygen sensitivity of the organism. It is not known whether growth with artificial iron chelators enhances the catalase ac-

tivity in S. volutans. The failure of even large inocula to grow aerobically in MPSS medium which had been stored in sunlight may be attributable to accumulation of peroxides in such medium. However, in view of the fact that added catalase did not permit aerobic growth of S. volutans (38), other explanations for the microaerophilism must be sought.

Quantities of other iron-containing cellular components, including succinic dehydrogenase and cytochromes, also have been shown to be lowered in iron-deficient cells (43). This finding may have significance for S. volutans in view of the data of Cole and Rittenberg (19), which indicate that all cytochromes, particularly cytochrome c, and also succinic dehydrogenase, were present in smaller amounts in this organism than in the two aerobic spirilla studied. These authors also reported that the activities of fumarase and malate dehydrogenase in S. volutans were very low; therefore, they pointed out, with succinate as substrate, succinic dehydrogenase is the major supplier of electrons to the respiratory chain. (The other two spirilla not only possessed higher activities of the enzymes mentioned, but also showed a high rate of oxygen consumption with peptone, completely lacking in S. volutans. Cole and Rittenberg suggested that the low level of succinic dehydrogenase may account for the low respiratory rate that they observed and may allow the accumulation of toxic levels of oxygen within the cells of S. volutans under an air atmosphere. Because succinic dehydrogenase is a sulfhydryl-containing enzyme, oxidation of the enzyme itself under these conditions may aggravate the deficiency.

In view of the above relationships, growth without iron chelators under microaerophilic conditions may be possible simply because less iron is required under these conditions for enzymes involved in the metabolism of, and defense against, oxygen.

Although lowered oxygen tension permitted growth of S. volutans in the absence of added iron chelators, the presence of such chelators and/or of ferrous iron did not permit growth from small inocula in shaken flasks of DBM under an air atmosphere. It is not certain, however, that these conditions were sufficient to supply adequate iron to the organism. Lankford (43) reported that growth of most organisms studied was stimulated much more strongly by their own endogenous chelating compounds than by "unnatural" substitutes. Perhaps filtrates from low-iron static cultures of S. volutans would be more effective than dihydroxyphenyl compounds.

Oxidation of the dihydroxyphenyl compounds themselves in aerobic cultures of S. volutans probably is enhanced by shaking, and any ferrous iron initially present undoubtedly is oxidized rapidly to the ferric state. In fact, the autoxidation of Fe^{++} generates the superoxide radical, which in turn is capable of oxidizing epinephrine to adrenochrome; the latter reaction was used as the basis for an assay of superoxide dismutase (23). These reactions should be considered in the light of the fact that growth under iron-deficient conditions has been used as a means of producing cultures of E. coli with low levels of the iron-containing superoxide dismutase of the periplasmic space (27). This enzyme has been shown to be a cellular defense against exogenously

generated superoxide, which may be produced in cultures of S. volutans by autoxidation of ferrous ions as mentioned above.

It seemed possible that, in static cultures, any iron chelator synthesized by the cells might accumulate to a locally high concentration in the vicinity of the cells, dispersing only by diffusion in the absence of shaking. Such a physical effect of shaking, however, does not in itself explain the inability of S. volutans to grow in aerobic cultures, because excellent growth was obtained from small inocula shaken in sidearm flasks under an atmosphere of 6% O_2: 94% N_2.

The initiation of growth from inocula of intermediate size under aerobic conditions in shaken flasks of DGM, followed by cessation of growth after 24 h at a turbidity of about 25 Klett units, is completely unexplained at present. Cole and Rittenberg (19) made a similar observation: "large inocula taken from young, motile cultures initially grew well even [modified MPSS] medium that had not been boiled [and cooled to expel dissolved O_2] , but growth often ceased after several generations." They also reported that when inocula of 4 X 10^3 cells were incubated in medium which had not been boiled, variable results were obtained in that "growth usually stopped after one or two cell divisions, but occasionally more extensive growth was observed." The growth observed in shaken cultures in DGM in the present study represented five or six cell divisions and was not improved by adding either succinate or additional iron chelators to the medium after 24 h. No plausible explanation is apparent for the cessation of growth once initiation has occurred.

The work of Cole and Rittenberg (19) and of Cole (18) raised several other points with regard to microaerophilism. The possibility of a low respiratory rate which would allow accumulation of intracellular oxygen, entering the cell by diffusion, to toxic levels has already been mentioned. It is noteworthy, however, that these investigators measured respiratory activity of cell suspensions in phosphate buffer, which they reported was inhibitory to respiration of S. volutans (19).

The low levels of fumarase and malate dehydrogenase reported in both of these investigations would leave succinic dehydrogenase as the only obvious source of electrons for the electron transport system of S. volutans. A decrease in the activity of this enzyme, whether because of iron deficiency or by oxidation of the sulfhydryl group, should be a serious disability for the organism when growing on succinate. However, Caraway and Krieg (14) obtained Q_{O_2} values with fumarate or malate as substrate which were approximately 70% of that obtained with succinate; apparently the enzymatic machinery does exist for utilization of these substrates.

Cole (18) concluded that the ability of S. volutans to initiate growth in media which had not first been boiled to expel dissolved oxygen was dependent on both the physiological state of the inoculum and its size. Data from the present study would seem to indicate, however, that inoculum size is of primary importance and that inocula of comparable size from various times in the growth curve, extending into the stationary phase, are capable of initiating growth in CHSS or in MPSS. Even one-loop inocula, adjusted to the same turbidity, initiated growth

after the usual lag in CHSS, regardless of the phase of growth of the culture from which they were taken.

Cole's hypothesis to explain the microaerophilism of S. volutans postulated the accumulation of oxygen within the cells after succinate had been exhausted. With no substrate available for the reduction of oxygen, the cells would die on continued exposure to oxygen but could remain viable if transferred to fresh, oxygen-depleted medium. In his experiments, cultures of S. volutans were first incubated under microaerophilic conditions for 12 h and then aerated; various metabolic parameters were measured after 8 and 12 h of aeration and compared to values for cultures maintained under microaerophilic conditions continuously for 18 h. The activity of succinic dehydrogenase did not vary greatly throughout the experiment. The activities of all other TCA cycle enzymes assayed (fumarase, malate dehydrogenase, aconitase, and isocitrate dehydrogenase) were much higher after 8 h of aeration than in microaerophilic cultures. Between the eighth and twelfth hours of aeration, however, the respiratory rate fell sharply, and fumarase and malate dehydrogenase activities dropped to undetectable levels. Cole reported that during this time succinate was exhausted. Thus, it is unclear whether the changes observed are attributable to the effect of oxygen, to lack of substrate, or to both of these causes. Because fumarase and malate dehydrogenase activities actually increased at least 4-fold during the first 8 h of aeration, it seems likely that substrate exhaustion and not oxygen toxicity caused their eventual decline. It is also true that not only inocula from stationary phase cultures in which

succinate has been exhausted, but also cells from cultures in logarithmic growth, are subject to inhibition by oxygen; exhaustion of substrate may aggravate microaerophilism but is not the cause of it.

The nature of the inhibition of S. volutans by phosphate and its relationship to oxygen toxicity still has not been elucidated. It appears that both phosphate toxicity and the need for exogenous iron chelators are related in S. volutans to oxygen toxicity; whether they are related to each other is not clear.

Weinberg (66) has described a relationship between the concentration of phosphate and secondary metabolism of bacteria. He defined secondary metabolites (63) as natural products which are restricted in taxonomic distribution and have no obvious function in cell growth, and which are synthesized by cells that have stopped dividing. While mentioning that many secondary metabolites are strong metal-binding agents, the yield of which may be controlled by concentrations of trace metals, he pointed out that iron chelators such as enterochelin and the mycobactins are not secondary metabolites. Although they may be present in large amounts during the stationary phase, they are produced during growth and, as has been discussed, during the lag phase. Lankford (43) reported that schizokinen synthesis ceases when cultures of B. megaterium enter exponential growth and is resumed during the late logarithmic and stationary phases.

Weinberg has proposed (65) that completion of secondary metabolism is a prerequisite for long-term survival of cultures of vegetative cells. In support of this hypothesis, he has presented data (66) showing that

high concentrations of phosphate, which inhibit secondary metabolism, also promote much more rapid cell death in cultures of six species of Gram-negative bacteria (Shigella, Salmonella, and Pseudomonas).

It is of interest to note the concentrations of phosphate referred to in the above report (66). Microbial growth in general is permitted, according to Weinberg, in media containing from 0.1 to 300 mM inorganic phosphate; but quantities greater than 1.0 to 10 mM inhibit secondary metabolism. In contrast, the growth of S. volutans in DGM under an air atmosphere was inhibited by phosphate concentrations as low as 10 mM; this organism is indeed sensitive to low concentrations of phosphate!

While an iron chelator which may be produced by S. volutans would not fall within the definition of a secondary metabolite, there are enough similarities to warrant the suspicion that its synthesis might be subject to inhibition by phosphate. If this were true, it might explain why phosphate is toxic to S. volutans if added to the medium when it is inoculated but not if added during the logarithmic phase of growth. If iron chelators are synthesized during the lag phase and synthesis declines or ceases during logarithmic growth, then the toxicity of phosphate would be exerted during the lag phase. In addition, because the need for iron chelators is less under microaerophilic conditions, the lesser toxicity of phosphate under these conditions might also be explained.

To summarize the results of this study with regard to Spirillum volutans, there are two nutritional factors which are known to affect the oxygen sensitivity of the organism. One is the sensitivity to

phosphate. The other is the apparent inability to synthesize chelators for ferric iron at a rate sufficient to permit initiation of growth from small inocula under aerobic conditions, at least without a very long lag phase. Whether these two factors are related to each other is not known, but numerous possibilities exist for interactions between phosphate sensitivity, iron metabolism, and microaerophilism.

The supposition that microaerophilism probably is not the result of a single factor is supported by these complex interactions. It is interesting to consider how an organism with these varied limitations survives in its natural habitat. S. volutans may be isolated from just beneath the scum which forms on the surface of a pond water hay infusion (68), and Ehrenberg (31; translation in 37) referred to the observation of this organism in "an infusion of vegetable garbage" and "ill-smelling infusions with a white film." Such environments should provide a variety of substrates and a large number of other bacteria which could reduce the oxygen tension. Because of the lowered oxygen tension, ferrous iron might be present. The other organisms may also provide chelating compounds capable of solubilizing ferric ions for S. volutans in a situation analogous to the existence of the siderochrome auxotrophs, Pilobilus and Arthrobacter, in dung and soil, respectively. These habitats are "siderochrome-containing environments" because of the presence of their other microflora (43).

The extent to which each of the specialized requirements of S. volutans contributes to the phenomenon of microaerophilism should now be amenable to investigation in the laboratory by manipulation of

single factors in defined growth medium.

Campylobacter fetus, in many ways, is remarkably similar to S. volutans. Both are Gram-negative, polarly flagellated, curved rods. The guanine + cytosine content of the DNA of Campylobacter is 30-35% (58), compared to 38% for S. volutans (38). Both organisms obtain energy by a strictly oxidative metabolism and are unable to utilize sugars, and both are obligately microaerophilic. It seemed possible, therefore, that a nutritional factor such as restricted ability to assimilate iron, which had been shown to be related to microaerophilism in S. volutans, might apply to C. fetus as well.

The streak plate experiments demonstrated that supplements of ferrous or ferric iron, or of o-dihydroxyphenyl iron chelators, not only stimulated microaerophilic growth of at least some strains of C. fetus, but also allowed excellent aerobic growth under an atmosphere enriched in CO_2 and adjusted to contain an oxygen tension comparable to that in the atmosphere at sea level. The ability to produce colonial growth, as pointed out by Halmann et al. (31), is a rigorous test of the adequacy of the conditions employed, because the inoculum cells are relatively isolated from the metabolic products of other cells in the inoculum which would be available to them during confluent growth or in broth cultures. (S. volutans, which does not form colonies on solid media even under microaerophilic conditions, could not of course be tested by this method.)

Excellent growth of C. fetus on plates of Albimi agar was obtained

with 0.05% added $FeSO_4 \cdot 7H_2O$, and 0.003% ferrous sulfate supported good growth of S. volutans in DBM. These concentrations are about 1.8 mM and 0.1 mM Fe, respectively. It is interesting to compare these levels of iron with Lankford's report (43) that maximal growth of most Gram-negative organisms occurs in deferrated media to which 0.36 to 1.8 μM Fe has been added. Clearly, both C. fetus and S. volutans are stimulated by iron at levels far exceeding that required for maximal growth of most bacteria.

The improved growth of C. fetus in medium containing added iron might be very useful in clinical microbiology laboratories. If many strains of this organism prove to be stimulated by added iron, the technique might provide a reliable method for isolation of C. fetus in an ordinary candle jar without replacing air with special gas mixtures containing 5% oxygen, as presently recommended (35). Bokkenheuser (4) noted that the number of reports in the world literature of isolations of C. fetus from human infections increased from about one per year in 1955 to about seven per year in 1970. He studied ten cases which were diagnosed in New York City within a period of 30 months and concluded that if this rate is representative of the worldwide occurrence of human infection with C. fetus, then fewer than 1% of actual cases are reported in the literature. According to Bokkenheuser, "It is clear that the principal obstacles to meaningful information are our inability to recognize the infection at the bedside as well as our deficient laboratory techniques for the isolation of Vibrio fetus. The solution of at least one of these problems is the key to further progress."

Data obtained in research with C. fetus concerning the influence of nutritional factors on microaerophilism may also be applicable to further studies with S. volutans. In some respects, C. fetus may be a better research tool for such studies, especially because it forms colonies on solid media and can therefore be exposed to atmospheres of known composition without the problem of diffusion limitation encountered in broth cultures. In addition, colonial growth facilitates enumeration of viable organisms to obtain quantitative results. The differences between the two organisms, however, must also be borne in mind. For example, phosphate toxicity does not seem to be applicable to C. fetus, which was not inhibited by the presence of 0.1% added phosphate (R. M. Smibert, personal communication).

The clear-cut results obtained with C. fetus, added to the data for S. volutans, lend support to the conclusion that iron metabolism may be a contributing cause of microaerophilism in bacteria.

SUMMARY

Two investigations were pursued in this study. One was a possible application of the motility of Spirillum volutans to detection of pollutants in industrial effluent for the purpose of in-plant biological monitoring. The other was a nutritional study of the organism with emphasis on its obligate microaerophilism.

In the pollution monitoring project, flagellar uncoordination in S. volutans was studied as a bioindicator of toxicity. Uncoordination is produced by a variety of agents. Cells displaying normal motility show frequent reversal of direction, accompanied by reversal of orientation of their bipolar fascicles of flagella. In uncoordinated cells, the flagella at opposite poles oppose each other and the cell cannot swim, although the flagella remain active.

A defined motility test medium was devised in an attempt to maximize the sensitivity of the uncoordination response to potential pollutants. Suspensions of S. volutans in this medium responded to a variety of agents, including metal ions (1-3 ppm), cetyl pyridinium chloride (1 ppm), hydrazine (10 ppm), 1-naphthol (3 ppm), and others. Effective concentrations ranged up to several per cent for the alcohols tested. The response was immediate.

A standardized method was developed which could be used to detect potential pollutants in industrial effluent before discharge, using motility of S. volutans as the indicator.

In the nutritional study, a defined growth medium was developed for S. volutans. The key to obtaining growth in a defined medium,

suggested by a "requirement" for autoclaved tyrosine, was the presence of a dihydroxyphenyl compound (such as arterenol or epinephrine) as a chelator for ferric iron, which was present at 3 mg/l $FeCl_3 \cdot 6H_2O$. Alternatively, growth occurred with high concentrations of ferrous iron. The defined medium also contained succinate, five amino acids, mineral salts, trace minerals, N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) buffer, and 10^{-4} M K_2HPO_4.

The ability to grow with ferric iron in the absence of an iron chelator was related to inoculum size; large inocula were able to initiate growth, while smaller inocula showed a long lag period or failed to grow. The inoculum-dependence probably was the result of impaired ability to produce an endogenous chelator for assimilation of ferric iron.

The presence of iron chelators improved aerotolerance of S. volutans, and growth from smaller inocula was initiated in the absence of a chelator if microaerophilic conditions (6% O_2: 94% N_2) were provided. Sensitivity to phosphate also was related to the presence of atmospheric oxygen; 10^{-2} M PO_4 prevented growth under an air atmosphere but not under microaerophilic conditions.

The stimulatory effect of iron chelators was applied to the cultivation of another obligate microaerophile, Campylobacter fetus, which is of clinical importance. This organism grew well on Brucella agar (Albimi) supplemented with 0.05% $FeSO_4 \cdot 7H_2O$ in a candle jar, or under full atmospheric oxygen if supplementary CO_2 was added. Without added iron, growth occurred only if the oxygen in the atmosphere was lowered

to about 9%. Ferric iron appeared to be as effective as the ferrous form in promoting growth of C. fetus. Dihydroxyphenyl compounds (arterenol and DOPA) permitted growth on Brucella agar in the absence of added iron.

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DEVELOPMENT OF DEFINED MEDIA FOR MOTILITY AND FOR GROWTH OF *SPIRILLUM VOLUTANS*, WITH SPECIAL REFERENCE TO BIOLOGICAL MONITORING OF POLLUTANTS AND TO OBLIGATE MICROAEROPHILISM OF BACTERIA

by

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(ABSTRACT)

Two investigations were pursued in this study. One was a possible application of the motility of *Spirillum volutans* to detection of pollutants in industrial effluent for the purpose of in-plant biological monitoring. The other was a nutritional study of the organism with emphasis on its obligate microaerophilism.

In the pollution monitoring project, flagellar uncoordination in *S. volutans* was studied as a bioindicator of toxicity. Uncoordination is produced by a variety of agents. Cells displaying normal motility show frequent reversal of direction, accompanied by reversal of orientation of their bipolar fascicles of flagella. In uncoordinated cells, the flagella at opposite poles oppose each other and the cell cannot swim, although the flagella remain active.

A defined motility test medium was devised in an attempt to maximize the sensitivity of the uncoordination response to potential pollutants. Suspensions of *S. volutans* in this medium responded to a variety of agents, including metal ions (1-3 ppm), cetyl pyridinium chloride (1 ppm), hydrazine (10 ppm), 1-naphthol (3 ppm), and others. Effective concentrations ranged up to several per cent for the alcohols tested. The response was immediate.

A standardized method was developed which could be used to detect potential pollutants in industrial effluent before discharge, using motility of S. volutans as the indicator.

In the nutritional study, a defined growth medium was developed for S. volutans. The key to obtaining growth in a defined medium, suggested by a "requirement" for autoclaved tyrosine, was the presence of a dihydroxyphenyl compound (such as arterenol or epinephrine) as a chelator for ferric iron, which was present at 3 mg/l $FeCl_3 \cdot 6H_2O$. Alternatively, growth occurred with high concentrations of ferrous iron. The defined medium also contained succinate, five amino acids, mineral salts, trace minerals, N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) buffer, and 10^{-4} M K_2HPO_4.

The ability to grow with ferric iron in the absence of an iron chelator was related to inoculum size; large inocula were able to initiate growth, while smaller inocula showed a long lag phase or failed to grow. The inoculum-dependence probably was the result of impaired ability to produce an endogenous chelator for assimilation of ferric iron.

The presence of iron chelators improved aerotolerance of S. volutans, and growth from smaller inocula was initiated in the absence of a chelator if microaerophilic conditions (6% O_2: 94% N_2) were provided. Sensitivity to phosphate also was related to the presence of atmospheric oxygen; 10^{-2} M PO_4 prevented growth under an air atmosphere but not under microaerophilic conditions.

The stimulatory effect of iron chelators was applied to the

cultivation of another obligate microaerophile, Campylobacter fetus, which is of clinical importance. This organism grew well on Brucella agar (Albimi) supplemented with 0.05% $FeSO_4 \cdot 7H_2O$ in a candle jar, or under full atmospheric oxygen if supplementary CO_2 was added. Without added iron, growth occurred only if the oxygen in the atmosphere was lowered to about 9%. Ferric iron appeared to be as effective as the ferrous form in promoting growth of C. fetus. Dihydroxyphenyl compounds (arterenol and DOPA) permitted growth on Brucella agar in the absence of added iron.