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CHARGE OF STAPHYLOCOCCUS AUREUS UNDER THREE
GASEOUS ENVIRONMENTS

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The effect of different oxygen atmospheres on growth, energy charge and enterotoxin production of Staphylococcus aureus was investigated. Three atmospheres, air, 5% oxygen in nitrogen and 20% oxygen in nitrogen, were tested on three strains producing enterotoxins A, B and C. Colony forming units (CFU), dry weight, dissolved oxygen, pH, adenosine phosphates and adenylate energy charge and enterotoxin production were measured. The effect of sugars added to the culture media was also studied.

A significant difference was observed among the atmospheres in their effect on growth in the earlier hours. Growth was fastest in air for all the strains, followed by 20% and 5% oxygen in nitrogen. Growth response of the strains to the atmospheres were similar among all three strains. Depletion of dissolved oxygen occurred with

rapid growth. The pH was alkaline in all cultures by the last hours sampled.

A significant difference was also observed among the atmospheres in the effect on enterotoxin production, although this differed among strains. In strains 265-1 and S-6, enterotoxin A and B production, respectively, was faster in air than it was in either 5% or 20% oxygen in nitrogen. This corresponded to the effect on growth rate. The response of strain 361 was distinctively different from the other two strains. In this strain the atmospheres of 5% and 20% oxygen in nitrogen had a stimulatory effect on the rate of production of enterotoxin C.

There was a definite pattern of change in energy charge during the growth cycle of S. aureus with apparent difference among three strains. In 265-1, the energy charge increased very rapidly and reached 0.68 at 3 hr of growth; an equivalent level was reached in strains S-6 and 361 at about 8 hr after an initial lag. The time at which enterotoxin production was detectable coincided with a rapid increase in energy charge, except with a lag for strain 361.

Added sugars had a slightly stimulatory effect on growth in air and a significantly stimulatory effect on growth in 5% and 20% oxygen in nitrogen. The decreased growth rate observed in 5% and 20% oxygen in nitrogen when cells were grown in the simple NAK medium was relieved in 5% and 20% oxygen in nitrogen when the sugars were added.

Sugars repressed enterotoxin production by S. aureus in air. However, the repressive effect of the sugars on enterotoxin production was not observed when the cells were grown in 5% and 20% oxygen in nitrogen. The relationship of energy charge to enterotoxin production should be studied with regard to the effects of media components.

Growth, Enterotoxin Production and Energy Charge
of Staphylococcus aureus under Three
Gaseous Environments

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GROWTH, ENTEROTOXIN PRODUCTION AND ENERGY CHARGE
OF STAPHYLOCOCCUS AUREUS UNDER THREE
GASEOUS ENVIRONMENTS

INTRODUCTION

Staphylococcal food-poisoning is the most commonly occurring form of foodborne disease in many countries of the world. The illness results from ingestion of food in which enterotoxins have been produced by Staphylococcus aureus. The foods that are usually involved in staphylococcal food-poisoning are cooked, protein-containing products which are contaminated by handling and allowed to remain in favorable environmental conditions for growth and enterotoxin production. In the United States, ham has been the most frequently involved food item in outbreaks during the years 1967 through 1973. Cream-filled pastry, poultry and beef are the next most frequently reported foods. Other foods that have been involved in outbreaks are fish and shellfish, processed meats, pork, milk and milk products, egg products, potato salad and macaroni salad (Center for Disease Control, 1968-1973).

Staphylococcal food poisoning occurs only when all of the following conditions are met (Bryan, 1969):

1. A source of an enterotoxigenic strain must be in the food preparation environment.
2. The organisms must be transferred from the source to a food.

3. The contaminated food must be capable of supporting the growth of S. aureus.
4. The contaminated food must remain in a favorable environment long enough to multiply and produce enterotoxin.
5. A sufficient amount of enterotoxin exceeding the susceptibility threshold of individuals must be ingested.

Control measures must eliminate at least one of the above conditions. The control of growth would be the most realistic approach because staphylococci are ubiquitous in man's environment, enterotoxins in food are stable to boiling water temperature and contamination and growth in foods are not easily detectable by sensory criteria. Only through knowledge of the effects of various environmental conditions on growth and enterotoxin production by staphylococci can proper food handling practices be developed.

Among these environmental factors, the effects of temperature, pH, salt concentration and water activity have been studied extensively and ranges of these factors for growth and enterotoxin production have been established. However, very little has been reported on the effect of the gaseous atmosphere. Barber and Diebel (1972) reported that no enterotoxin was produced in inoculated sausages during anaerobic incubation for 120 hr although significant growth had occurred. However, no investigation has been done specifically on the effect of oxygen on growth and enterotoxin production.

The purpose of this research was to investigate the effect of

oxygen on the growth and enterotoxin production of S. aureus. Three gaseous atmospheres, 5% oxygen in nitrogen, 20% oxygen in nitrogen and air were investigated. These three were chosen in order to study the effect of oxygen level and other components of air on growth and enterotoxin production of three strains of enterotoxigenic staphylococci. The adenylate energy charge, a recent concept for measuring the metabolically available energy, was measured in order to probe into the energetics of staphylococcal metabolism during growth and enterotoxin production. The effect of simple sugars added to the standard medium on growth and enterotoxin production was also investigated to observe the interaction between oxygen availability and readily available energy source in the medium.

REVIEW OF LITERATURE

Staphylococcal enterotoxins

Enterotoxins are extracellular proteins produced by staphylococci under certain conditions in foods or in culture media. They are single, unbranched polypeptides of molecular weight of 30,000 to 35,000 (Bergdoll, 1972). Ingestion of these substances causes staphylococcal food-poisoning, the most common foodborne illness in many parts of the world.

At least five antigenically distinct enterotoxins have been identified and have been labelled type A, B, C, D and E. A single strain may produce two or more enterotoxins. Type A is the most commonly involved in food-poisoning outbreaks, followed by type D and type C. Type B is known to be seldom implicated (Casman et al., 1967; Baird-Parker, 1971; Bergdoll, 1972).

Dornbusch et al. (1969) investigated the genetic nature of enterotoxin production using type B producing strains and proposed that the production of enterotoxin B is under extrachromosomal control. Treatment of cells with acriflavin, which is known to eliminate extrachromosomal determinants, resulted in the elimination of enterotoxin production as well as methicillin and mercury resistance. It was also proposed that enterotoxin production may be under the control of a single plasmid. In their transduction experiments, enterotoxin B

production was transferred at the same frequency as methicillin resistance.

A number of investigators have postulated that the pathway of biosynthesis of enterotoxin A is distinct from that of enterotoxin B. The first definitive study was reported by Markus and Silverman (1970). In their earlier study (1968), it was found that 95% of enterotoxin B was released at the latter part of the exponential and the beginning of the stationary phase of growth. In their later study (1970), they found that enterotoxin A, unlike enterotoxin B, was released during the exponential phase. They proposed that enterotoxin A is a primary metabolite and B is a secondary metabolite. However, in a more recent study by Czop and Bergdoll (1974), it was observed that enterotoxins A and B were both produced during all phases of growth. The difference was in the rate of synthesis; the exponential rate of synthesis was much greater for enterotoxin B than it was for A.

Evidence that production of enterotoxin may differ is presented in a study of L-forms obtained from strains producing enterotoxins A, B and C (Czop and Bergdoll, 1970). Only L-forms from A strains produced enterotoxin; those from B and C did not. From these observations, they concluded that enterotoxin B and C production is cell surface oriented, whereas enterotoxin A is not. Friedman (1968) reported a study that supports the hypothesis that enterotoxin B

production is associated with the cell surface. It was observed that the formation of enterotoxin B was inhibited by detergent-like compounds such as Tween 80, oleic acid and sodium deoxycholate, and also by compounds that block cell wall synthesis, such as penicillin and bacitracin.

The amount of enterotoxin produced in laboratory media varies among strains. The type B-producing strains are known to give the highest amount of enterotoxin with a maximum reported value of 578 μg per ml (Metzger et al., 1973), followed by 100 μg for type C-producers (Genigeorgis et al., 1971b), and 50 μg for type A producers (Friedman and Howard, 1971).

Data concerning the amount of enterotoxin needed to cause symptoms in humans are very limited. Baird-Parker (1971) reported that the average emetic dose of enterotoxins A, B and C is about 0.2 μg per kg body weight for adults. Raj and Bergdoll (1969) reported that 0.4 μg of pure enterotoxin B per kg body weight produced clinical manifestation in three human volunteers. Evidence from foods involved in a food-poisoning outbreak indicates that the dose for enterotoxin A may be much smaller (Bergdoll, 1970). In one outbreak, the person that became ill after eating the smallest amount of contaminated cheese had received less than 1 μg of enterotoxin A.

Although a number of investigators have attempted to understand the mechanism of enterotoxin production, very few theories

have been proposed and it is very far from elucidation. Numerous studies have been reported on extracellular factors affecting the production of enterotoxin by staphylococci (Baird-Parker, 1971; Bergdoll, 1972; Minor and Marth, 1972a). However, environmental factors are very much interrelated in exerting their effects and strains differ in their response to environmental conditions. Therefore, judgment should be exercised in interpreting the studies on individual factors or on individual strains.

Factors affecting enterotoxin production

It has been known that enterotoxin production is a function of environmental conditions as well as genetic potential. Growth and enterotoxin production by S. aureus are influenced by temperature, pH, salt concentration, type of medium, water activity, type of atmosphere and competing organisms. All of these factors are interrelated in their effects. However, it is important to investigate each factor individually in understanding the total effects of the above factors and their interrelationships.

Nutrient medium. Most widely used media for growth and enterotoxin production are meat infusion broths such as veal, beef, beef heart and brain-heart and simplified media consisting of casein hydrolysate such as Protein Hydrolysate Powder (PHP) (Mead Johnson, Evansville, Ind.) and/or N-Z Amine (NAK) (Humko Sheffield

Chemical, Lyndhurst, N.J.) (Ashton and Evancho, 1973). It is a general practice to supplement the simplified medium with thiamin and niacin for obtaining high yields of enterotoxins (Kato et al., 1966). Niacin and thiamin are known to be essential for the growth of S. aureus; niacin is needed for glycolysis and thiamin in pyruvic oxidation (Kligler et al., 1943).

The effect of the medium on the production of enterotoxins can be considerable; however, there is no general consensus among investigators concerning the best medium for enterotoxin production (Bergdoll, 1972). Bergdoll and coworkers at the Food Research Institute use 3% PHP + 3% NAK for production of all enterotoxins (Bergdoll, 1972). Casman and Bennett (1963) recommended Brain Heart Infusion (BHI) medium. Mullet and Friedman (1961) reported that BHI was a poor medium for the production of enterotoxin B. However, Genigeorgis and Sadler (1966) obtained good yields of enterotoxin B using BHI.

It is known that the source of the protein ingredient can affect enterotoxin production. Reiser and Weiss (1969) found that Difco BHI was inferior to either Fisher BHI, 4% NAK, or 3% NAK + 3% PHP for the production of enterotoxins B and C. Metzger et al. (1973) reported that a maximum amount of enterotoxin B (578 $\mu\text{g}/\text{ml}$) was obtained using 4% NAK (Sheffield) enriched with 0.2% glucose and 1% yeast extract.

Great effort has been made by a number of investigators toward the formulation of synthetic media for growth and enterotoxin production, since it is desirable to select the simplest medium available for the subsequent purification of enterotoxins (Bergdoll, 1972). Surgalla (1948) investigated various synthetic media consisting of amino acids, inorganic salts, vitamins and glucose. Enterotoxin was produced in media containing 2 to 16 amino acids. The simplest medium permitting enterotoxin production contained two amino acids, arginine and cystine, and glucose. Growth was not observed in a medium containing three amino acids (cystine, arginine and glycine) when glucose was absent.

The fact that arginine and cystine are essential amino acids for staphylococcal growth and enterotoxin synthesis was also reported by Peters (1964). In his synthetic medium consisting of glucose, ammonium sulfate, and other inorganic salts, thiamin and niacin and amino acids, it was observed that the above two amino acids were minimum requirements for all six strains studied. More amino acids were required (up to eight) when glucose was absent from the media. In the absence of glucose, enterotoxin was produced only after prolonged incubation (72 hr). It was his opinion that glucose is not essential for production of enterotoxin.

Temperature. The optimum temperature for growth and enterotoxin production is 37 C. The lowest temperature reported as

permitting staphylococcal growth is 6.7 C and the maximum is 47.8 C. Angelotti et al. (1961) observed staphylococcal growth in some foods at 6.7 C and George et al. (1959) observed growth at 47.8 C in condensed skim milk. The temperature range for enterotoxin production is slightly narrower than that for growth. Genigeorgis et al. (1969) reported that the lowest temperature permitting toxin production by strain S-6 in cured meats incubated anaerobically for up to 16 weeks was 10 C. The upper temperature limit is 45-46 C (Tatini et al., 1971a; Scheussner et al., 1973).

At extremes of temperature, enterotoxin production is greatly reduced (Scheussner and Harman, 1971; Marland, 1966) and delayed at lower temperatures. Marland (1966) reported that strain S-6 in PHP medium produced 320 µg/ml of enterotoxin B at 37 C in 24 hr, 60 µg/ml in 24 hr at 43 C and 2 µg/ml in 168 hr at 15.2 C. However, Tatini et al. (1971a) observed that enterotoxin production was stimulated in strains A, B, C and D by incubating at higher temperatures in BHI broth. The stimulative effect was greatest at 45 C followed by 40 and 37 C in all strains.

Some strain differences were observed in temperature response among strains by Scheussner et al. (1973). At 45 C and in a BHI medium, strains producing types A, C and D grew and produced enterotoxin, but there was a rapid decrease in viable cells and no enterotoxin was produced by a B strain. All strains grew and produced

enterotoxin at 19-29 C. At 7 C and at 50 C, none of the strains grew. At 13 C, A, B and C strains grew, but only the B strain produced enterotoxin; the strain producing D did not grow.

McLean et al. (1968) observed a reduction in the amount of enterotoxin B produced by strain 243 as the incubation temperature was decreased below 37 C. After 112 hr in BHI broth at 16, 20 and 37 C, the same growth was obtained but the amount of enterotoxin produced per ml was 8, 20 and 340 µg, respectively.

pH. There is a definite strain difference in response of S. aureus to pH. Barber and Deibel (1972) observed in their study with 13 different strains producing enterotoxins A, B, C and E that the lowest initial pH values for enterotoxin production in BHI ranged from 4.8 to 5.4. Strains producing enterotoxins B and C were influenced to a greater extent by pH than type A producers. Reiser and Weiss (1969) found that an initial pH of 6.8 gave higher yields of enterotoxins B and C than either 6.5 or 5.3, while the production of enterotoxin A was not affected by an initial pH of 5.3. Kato et al. (1966) also reported that initial pH values in the range of 5.0 to 8.0 did not affect enterotoxin C production. Tatini et al. (1971b) reported enterotoxin A production in reconstituted non-fat milk solids with an initial pH as low as 4.5.

The pH range for good growth and enterotoxin production of S. aureus extends from 5.0 to 8.0 (Bergdoll, 1972 and

Peters, 1964). The minimum pH for initiation of growth was 4.0 and the maximum was 9.83 in a study with strain 137 which produced enterotoxin C in PHP-NAK broth (Genigeorgis et al., 1971a). Peters (1964) observed a complete inhibition of growth and enterotoxin production below a pH of 4.1 to 4.6 and above 8.5 to 9.5, depending upon medium and strain.

In a study of enterotoxin B production in pH-controlled NAK medium, Metzger et al. (1973) found that control at 7.0 gave the highest production, alkaline control (pH 8.0) gave a very minimal amount, and acid control (pH 6.0) resulted in 50% reduction. Slightly less enterotoxin was produced when pH was not controlled than when pH was held at 7.0.

The type of acid used is known to influence the response of S. aureus. Nunheimer and Fabian (1940) found that the decreasing order of germicidal effect was acetic, lactic, tartaric and hydrochloric acids. Minor and Marth (1972b) investigated the survival of strain 100 in Trypticase Soy Broth acidified with five different acids. When the organisms were incubated in the media adjusted to pH values of 5.2-3.6, 90-99.9% of the cells were inactivated. Acetic, lactic and perchloric acids were more active in inactivation than was hydrochloric acid, and citric acid was equal to hydrochloric acid. It was also observed that the cells were more susceptible to inactivation by acids at a high incubation temperature. Temperatures between 10 and

37 C had no effect on inactivation of the cells by the acids. At 45 C the loss of viability was enhanced almost 1000 times.

Salt concentration in the medium is known to affect the response of the cells to pH. Genigeorgis and Prucha (1971) found in their study that the pH range for enterotoxin C production decreased as the salt concentration in the PHP-NAK broth increased; at 0% salt, the pH range was 4.0-9.83; at 4% salt, 4.40-9.43 and at 10% salt, 5.54-7.30.

Atmosphere is also known to influence the response of staphylococci to pH. Barber and Diebel (1972) reported that production of enterotoxin A is more acid tolerant aerobically than anaerobically. Most of the A strains tested produced detectable enterotoxin aerobically at pH 5.1 but not below pH 5.7 anaerobically.

Other microorganisms. The presence of other microorganisms in the culture medium is known to influence staphylococcal growth and enterotoxin production. Although there are numerous reports on the effect of competing microorganisms on growth, very few studies involved the effect on enterotoxin production (Baird-Parker, 1971).

Graves and Frazier (1963) screened 870 cultures of organisms isolated from market food samples before and after storage at different temperatures. About 50% of the cultures were found to affect the growth of staphylococci. More than one-half of the effective cultures were inhibitory and less than half were stimulatory.

Kao and Frazier (1966) studied the effect of bacteria commonly

found in foods on staphylococcal growth. They observed that some of the organisms such as Streptococcus faecalis, S. faecium, Lactobacillus lactis, L. brevis and Leuconostoc mesenteroides stimulated the growth of S. aureus during the early hours, especially at higher temperatures, but most bacteria were inhibitory and some were even bactericidal. Lactic acid bacteria gave more complicated results; some were consistently inhibitory, some were inhibitory at lower temperatures, some were stimulatory at all temperatures and some were stimulatory at higher temperatures only.

McCoy and Faber (1966) extended their study of the effect of other microorganisms to enterotoxin production. Forty-four common food bacteria were investigated for their influence on 15 strains producing enterotoxin A. There were more organisms that were inhibitory than were stimulatory. Of the 44 organisms studied, only Bacillus cereus was significantly stimulatory to growth and enterotoxin production. They observed two types of inhibition: inhibition of growth and enterotoxin production and inhibition of enterotoxin production without apparent effect on growth. Pseudomonas sp. MID-1, Salmonella enteritidis and S. plymuthica markedly inhibited both growth and enterotoxin production and Serratia marcescens inhibited only enterotoxin production.

Curing salts. The presence of salts in the medium affects both growth and enterotoxin production of S. aureus. Although staphylo-

cocci will grow at salt concentrations up to the saturation level (25%), above 10% growth is diminished (Parfentzer and Catelli, 1964).

Genigeorgis and Riemann (1973) observed that S. aureus grew up to 16-18% salt concentration aerobically and 14-16% anaerobically.

The maximum salt concentration permitting enterotoxin production is lower than that for growth. Baird-Parker (1972) stated that salt concentrations of up to 5% did not affect enterotoxin B production by a S-6 strain grown in PHP medium; however, the amount of enterotoxin produced was diminished above 7% although the growth was not affected up to 10% salt concentration. McLean et al. (1968) also observed that salt concentrations of up to 10% had very little effect on growth, but above 3% the amount of enterotoxin B produced was decreased.

Markus and Silverman (1970) reported that concentrations of salt up to 10% did not affect the production of enterotoxin A by strain 100. Genigeorgis et al. (1971a) found that enterotoxin C production was not affected by salt concentrations of up to 10%.

Genigeorgis and Sadler (1966) found that enterotoxin B was produced at an initial pH of 6.9 in the presence of up to 10% salt and at an initial pH of 5.1 in the presence of up to 4% salt during incubation for 10 days at 37 C in BHI medium. In a later study (1971b), they observed that the pH range for growth of strains producing A, B, C and D decreased as the salt concentration in the medium increased.

The same trend of response was observed in the production of enterotoxin C (1971a). The range of initial pH permitting enterotoxin production decreased as the salt concentration increased. The range of pH was 4.00-9.83 at 0% salt and 5.44-7.3 at 10% salt. No enterotoxin was detected at pH 4.5-8.5 when 12% salt was present in the medium.

Curing salts such as sodium nitrate and sodium nitrite in maximum concentrations permitted in cured meats (1,000 ppm and 200 ppm, respectively) were found to have no effect on growth and enterotoxin production of strains producing A, B and C (Markus and Silverman, 1970; McLean et al., 1968; Genigeorgis and Prucha, 1971).

Water activity. Scott (1953) was the first to study the effect of water activity (a_w) on the growth of S. aureus. In 14 strains of food-poisoning staphylococci, he observed that growth rates were uniformly reduced as the a_w of the medium was lowered. The minimum a_w permitting staphylococcal growth was 0.86. At this a_w , growth was not observed anaerobically.

Troller (1971) studied the effect of a_w on growth and enterotoxin B production by strain C-243. The rate of growth was diminished at low a_w levels, and enterotoxin production was extremely sensitive to reduction in a_w . A reduction of a_w from 0.99 to 0.98 in one medium and from 0.99 to 0.97 in the other resulted in reduction of enterotoxin production by 90 to 99%, although the growth was not affected. In a

second study (1972), Troller investigated the effect of a_w on growth and enterotoxin A production by strain 196E. The growth response was similar to that of the enterotoxin B producer; however, the production of enterotoxin A was affected less by the decrease in a_w . A reduction of a_w from 0.97 to 0.945 resulted in a 60% decrease in the amount of enterotoxin produced. Troller suggested that the higher sensitivity of enterotoxin B producers to the level of water activity may be one of the reasons for the rarity of outbreaks of foodborne illness caused by enterotoxin B. Many of the foods which support enterotoxin production do not exceed a_w levels of 0.95 to 0.96.

Atmosphere. Although it has been known that the atmosphere influences growth and enterotoxin production by S. aureus, very few studies reported have been concerned specifically with the gaseous environment. Most of the studies involve only the comparison between aerobic and anaerobic conditions.

Aerobic cultures produce enterotoxin more rapidly and in larger amounts than do anaerobic cultures (Barber and Diebel, 1972; McLean et al., 1968). Baird-Parker (1971) found that after incubation for 7 days at 30 C the amount of enterotoxin B in static culture was 10 times higher for cultures incubated in the presence of air than for cultures incubated in a 95:5 mixture of nitrogen:carbon dioxide. Morse and Baldwin (1973) reported that the amount of enterotoxin B produced aerobically is 40 times higher than that produced

anaerobically.

Christianson and Foster (1965) reported that vacuum packaging of sliced ham resulted in a marked inhibition of the growth of S. aureus. When the inoculated hams were incubated, the non-vacuum packaged hams contained twenty times more organisms than the vacuum packaged hams.

Thatcher et al. (1962) studied the effect of various gaseous conditions on growth and enterotoxin production. They reported that S. aureus grew profusely and produced enterotoxin under atmospheres of air, 5% CO₂ in oxygen and 100% nitrogen. Only small amounts of enterotoxin were produced under vacuum. They stated that the mixture of CO₂ and O₂ was used because experience had shown this to be a suitable atmosphere for enterotoxin production.

Watt (1973) reported that the addition of 10% CO₂ in anaerobic incubation jars increased the colony size and the colony forming units in obligate and facultative anaerobes; however, S. aureus was not studied.

Role of oxygen in bacterial metabolism

Functions. One of the major functions of oxygen in micro-organisms is its role as an electron acceptor in the electron transport system of oxidative phosphorylation. Oxygen accepts electrons from terminal oxidases of the respiratory chain and is reduced to water or

hydrogen peroxide. Cytochrome oxidases are the most common oxidases; however, other types such as flavoprotein oxidases are also known (Dolin, 1961; Hughes and Wimpenny, 1969).

Oxygen also performs a role of nutrient in some bacteria. In the majority of microorganisms, most of the cell oxygen is derived from carbon dioxide by fixation reactions such as photosynthesis or from water by reactions such as those of NAD-catalyzed aldehyde dehydrogenase. However, it is known that in some specialized bacteria, such as those which utilize hydrocarbons as a carbon source, the molecular oxygen is incorporated into cell constituents by the reactions of oxygenases (Goldfine, 1965). Using $^{18}\text{O}_2$, Higgins and Quayle (1970) found that in *Pseudomonas methanica* and *Methanomonas methanoxidans* a large proportion of the cellular oxygen was derived from molecular oxygen. Microbial enzymes that activate molecular oxygen such as monooxygenase and dioxygenase have been identified and isolated from a number of hydrocarbon-utilizing organisms (Hughes and Wimpenny, 1969). In some yeasts and fungi, molecular oxygen is known to be incorporated into unsaturated fatty acids and sterols (Goldfine, 1965).

Oxygen plays an important role as a regulator of metabolism. Some enzymes are induced and some are repressed by the presence of oxygen. The production of yellow pigment in *Klebsiella aerogenes* is induced by oxygen (Harrison et al., 1969). Jacobs et al. (1967)

observed in S. epidermidis that respiratory enzymes and cytochrome pigments were controlled by the availability of oxygen. Molecular oxygen is known to be required for the induction of cytochromes in some yeasts (Harrison, 1972). Oxygen also induces the formation of many respiratory dehydrogenases and TCA cycle enzymes (Hughes and Wimpenny, 1969).

Response of bacteria to oxygen. Response of bacteria to the level of dissolved oxygen varies greatly ranging from obligatory anaerobes to obligatory aerobes. Those organisms that are dependent on aerobic respiration for energy metabolism and for which molecular oxygen functions as a terminal oxidizing agent are called obligatory aerobes. At the other physiological extreme are those microorganisms that do not require molecular oxygen. For many of these organisms, oxygen is a toxic substance which either kills them or inhibits their growth. Such organisms are obligatory anaerobes.

Some microorganisms are facultative anaerobes (or facultative aerobes) which can grow either in the presence or in the absence of oxygen. Some microbiologists (Stanier et al., 1970) divide facultative anaerobes into two categories. Some, like the lactic acid bacteria, have an exclusively fermentative energy-yielding metabolism but are not sensitive to the presence of oxygen. Others, such as many yeasts, coliform bacteria and S. aureus, can shift from a respiratory to fermentative mode of metabolism. Such facultative anaerobes use

oxygen as terminal electron acceptor when it is available but can also obtain energy in its absence by fermentative reaction.

The injurious effect of traces of oxygen on anaerobic bacteria is the most explicit example of oxygen toxicity. However, even aerobic organisms can be inhibited by oxygen in sufficiently high concentrations. Caldwell (1965) reported that growth was completely suppressed when bacterial and fungal cultures were incubated at 10 atm oxygen. On return to normal air, the organisms resumed normal growth. The inhibitory effect of hyperbaric oxygen for facultative anaerobes was reported by Towers and Hopkins (1965). They observed an inhibition of growth when staphylococci were intermittently exposed for 6 to 8 hr to 2 atm oxygen.

The mechanisms for oxygen toxicity have not been elucidated. The reason may be that they are very diverse. Various theories have been proposed. Some investigators attribute the toxic effect of oxygen on obligate anaerobes to the formation of hydrogen peroxide which cannot be removed in the absence of catalase (Harrison, 1972). Dolin (1961) attributed oxygen toxicity on anaerobic microorganisms to the autoxidation of cytochromes. Hyperbaric oxygen toxicity has been attributed to oxidation of thiol groups, enzyme inactivation, lipid peroxidation and free radical formation (Haugaard, 1968; Harrison, 1972; Hewitt, 1950).

Microorganisms use oxygen, oxygen donor, or electron acceptor

to utilize nutrients. A definite redox potential develops in each culture. This redox potential results from the difference between the electron attraction of the electron acceptors and the electron pressure of electron donors. Redox potential in bacterial cultures falls soon after the fresh subculture is made. A change of the redox potential to a more negative value indicates the start of growth. The redox potential becomes lowest in the exponential growth phase. During this period the organisms exhaust all the free oxygen in the medium (Hewitt, 1950).

At the stationary phase the potential becomes more positive due to the diminished metabolism. When the phase of active proliferation of organisms is over and the rate of dying of the bacteria exceeds the rate of multiplication, the lysis of bacteria liberates reducing substances and a more negative potential results. The rate and extent of changes in potential depend on the growth rate and the physiological type of the microorganisms.

Essentially, redox potential is an indirect measurement of the effect of electron acceptors on products of metabolism (Wimpenny, 1969). Therefore, the redox potential is influenced by the availability and the level of oxygen in the medium. Jacobs et al. (1967) conducted comparative measurements of redox potential and oxygen pressure in nutrient solutions and found that the redox potential is proportional to the logarithm of oxygen pressure. However, the correlation was shown

for only a narrow range of redox potential.

Since the redox potential of culture solutions can be controlled by measured introduction of oxygen, some investigators advocate its use as a control for aeration of bacterial cultures in oxygen-limited growth conditions. Wimpenny (1969) stated that redox potential may be profitably used to control aeration at oxygenation rates that are too low to be measured using an oxygen electrode in the growth medium.

Redox potential is also influenced by the pH of the culture medium; therefore, values for redox potential should be accompanied by a statement of the pH at which they were measured. In general, a pH variation of one unit causes the potential change of 57.7 mV (Jacob, 1969).

Besides oxygen and pH, the redox potentials of a culture are affected by other factors such as the chemical composition of the medium and the metabolic products. It is almost impossible to comprehend what substances are responsible for these potentials and what reversible oxidation-reduction systems are actually being measured. Therefore, the concept of overall redox potential is considered to be of little value for the study of growing microbial cultures (Harrison, 1972).

Effect on metabolism of *Staphylococcus aureus*. Most of the studies on carbohydrate metabolism have been done with glucose. It

has been known for several decades that staphylococci metabolize glucose aerobically as well as anaerobically via the Emden-Meyerhof (EM) glycolytic pathway. The predominant end products of aerobic glucose metabolism are acetate and carbon dioxide while lactic acid is the major end product of anaerobic metabolism (Blumenthal, 1972).

With the emergence of the use of specifically labeled ^{14}C glucose, many investigations were done on the quantitative analysis of the pathways of glucose metabolism in staphylococci. However, agreement concerning the metabolic pathways has not been attained since there have been no systematic studies of the effects of various physical and physiological conditions.

Glucose is metabolized via both EM and HMP (hexose monophosphate shunt), the extent to which each pathway is utilized depending on various factors. Initial pH of the medium is known to influence the pathway; the EM pathway being more active at high pH and the HMP pathway at low pH. Previous growth conditions are also known to affect the pathway; the EM pathway being more active in glucose-grown cells while the HMP pathway is more active in casein hydrolysate-grown cells. The addition of different nutrients to the medium also affects the extent of the pathway used. The addition of glucose is known to reduce the use of the HMP pathway by 50%. Niacin doubles the use of the HMP pathway, while thiamin has no significant effect (Blumenthal, 1972).

The oxygen tension of the growth medium influences the pathway used. During anaerobic growth, the HMP pathway is only half as active as that observed in cells grown aerobically. The anaerobic growth condition is known to have a much more profound effect on the subsequent activity of the TCA (tricarboxylic acid) cycle, inhibiting it up to 95% (Montiel and Blumenthal, 1965). After an extensive review of rather conflicting reports on the studies of glucose metabolism of staphylococci, Blumenthal (1972) concluded that in staphylococci, as in most biological systems, the EM pathway is the major one used for energy production while the HMP pathway mainly serves for production of biosynthetic precursors.

Very little has been reported on the protein metabolism of staphylococci. Except for a few reports on indirect evidence of amino acid assimilation and protein synthesis, no pathway of protein metabolism has been elucidated. Study of the effect of oxygen on protein metabolism is limited. Kendall and coworkers (1930) observed that resting cells of S. aureus utilized alanine much more in the presence of oxygen than in its absence.

Very little is known about lipid metabolism in microorganisms in general. Staphylococci produce a variety of phospholipases, lipoprotein lipases, esterases and lyases. Lipids, Tween, Span and phospholipoproteins are hydrolyzed with the release of fatty acids (Baird-Parker, 1972).

Oxygen is known to be essential for the synthesis of unsaturated fatty acids in yeasts. Bloomfield and Bloch (1960) reported that molecular oxygen is required for the desaturation of palmitic to palmitoleic acid and of stearic to oleic acid in Saccharomyces cerevisiae. They observed that artificial electron acceptors could not replace the function of oxygen. Babij et al. (1969) reported that a high oxygen concentration increased the level of polyunsaturated fatty acids in Candida utilis. Frerman and White (1967) observed that the total phospholipid content of the cells increased two-fold when an anaerobically grown S. aureus culture was shifted to an aerobic growth condition.

Effect on growth. In the case of obligatory aerobes and anaerobes, the effect of oxygen on growth yield (weight of cells per mole of substrate utilized) is simple and absolute, but in facultative organisms the situation is more complicated (Hughes and Wimpenny, 1969).

Facultative organisms such as staphylococci can proliferate both aerobically and anaerobically; however, growth is more efficient and faster aerobically than it is anaerobically. This effect of oxygen on the growth of microorganisms has been attributed to the fact that growth is related to the yield of ATP in microbial metabolism. Bauchop and Elsdon (1960) observed that the growth yield of microorganisms was proportional to the yield of ATP and estimated that one mmole ATP was needed to produce 10 mg cells. The efficiency

of aerobic growth can be easily comprehended when aerobic and anaerobic ATP production is compared.

Adenosine phosphates and energy charge. Quantitation of ATP and adenylate energy charge may be used to indicate different aspects of a bacterial culture. The amount of ATP seems to be an excellent measure of the concentration of living material. It is being used for determination of biomass in waters and as a means of enumerating bacteria in urine and in blood in medical laboratories (Cheer et al., 1964). Sharpe et al. (1970) reported that ATP determination can give a better estimate of biological contamination in foods than can the conventional plate count.

The adenine nucleotides, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), are known to stoichiometrically couple the metabolic sequences of a living cell (Chapman et al., 1971). The amount of metabolically available energy that is momentarily stored in the adenylate system was found to be linearly related to the mole fraction of ATP plus half the mole fraction of ADP (Atkinson and Walton, 1967). This parameter has been termed energy charge; in terms of concentrations, the energy charge is $(ATP) + \frac{1}{2} (ADP) / (ATP) + (ADP) + (AMP)$ (Atkinson and Walton, 1967; Atkinson, 1968; Atkinson, 1969).

The energy charge modulates the activity of various metabolic sequences related to energy utilization and regeneration. In bacteria

ATP-utilizing enzymes increase their activities when the energy charge is greater than 0.75 and ATP-regenerating enzymes predominate at an energy charge lower than 0.75 (Chapman et al., 1971).

The adaptability of facultative organisms to oxygen availability has been attributed to two levels of control. One is the long-term control which is exerted by induction and repression of enzymes and cytochrome synthesis. The other is the short-term control which is effected by adenine nucleotides which interact with anabolic and catabolic pathways and control the amount of substrate oxidized and the amount used for cell materials. Presumably a high level of ATP favors anabolism and a low level favors catabolism (Harrison et al., 1969; MacLennan and Pirt, 1970).

Chapman et al. (1971) have tabulated the published estimates of energy charge values in a number of microorganisms. No data, however, have been published concerning adenosine phosphate concentrations and the adenylate energy charge in S. aureus.

Relation to enterotoxin production. Aerated cultures of S. aureus produce toxin more rapidly and in larger amounts than do static cultures. Virtually all methods for producing enterotoxin make a provision for aeration or exposure to atmospheric oxygen (Baird-Parker, 1972; Minor and Marth, 1972a).

However, very little investigation has been done on quantitative aspects of oxygen in enterotoxin production. Marland (1966) in a

study of the effect of oxygen and aeration rate on enterotoxin B production found that certain oxygen and aeration rates were optimal and that excessive aeration or oxygen levels decreased the amount of enterotoxin produced without affecting the growth rate. No enterotoxin was detected in anaerobic cultures although growth was fairly good.

Markus and Silverman (1968) reported that an aerobic condition is essential for enterotoxin B synthesis by non-growing cells harvested from the later part of exponential and early stationary phases of growth.

MATERIALS AND METHODS

Three strains of S. aureus were used in the first experiment, which was based on growth in NAK medium. Each strain was grown under three different oxygen environments: air, 5% oxygen + 95% nitrogen, and 20% oxygen + 80% nitrogen. Three replications were conducted. Each replication required two series of experiments: the first series consisted of sampling hours of 3, 5, 7, 11 and 16 hr and the second series of 7, 11, 16, 20 and 24 hr. Analyses involved colony forming units (CFU), dissolved oxygen (DO), pH, adenosine phosphates and energy charge and enterotoxin. In the second experiment, three sugars were added to the NAK medium and the effect on growth and enterotoxin production during growth in the three atmospheres were studied using one strain. Three replications were done with sampling hours of 3, 5 and 7.

Cultures

S. aureus strain 265-1, a strain that produces enterotoxin A, was obtained from R. W. Bennett (Food & Drug Administration, Washington, D. C.); strain S-6 that produces enterotoxin B, from the Department of National Health and Welfare, Canada; and strain 361 producing enterotoxin C, from M. S. Bergdoll (Food Research Institute, University of Wisconsin). The cultures were maintained on porcelain beads over desiccant at 4 C (Hunt, Gourevitch and

Lein, 1958). In the first experiment, the three strains were used. In the second for effect of sugars, only strain 265-1 was used.

Inoculation

Inoculum was prepared by incubating beads in BHI medium (Difco Laboratories, Detroit, Mich.) for 24 hr at 37 C. For growth and enterotoxin production, a 50 ml portion of 4% N-Z-Amine Type NAK (Humko Sheffield Chemical, Lyndhurst, N. J.) supplemented with 50 µg of thiamin per ml and 1 mg of niacin per ml in a 250-ml Erlenmeyer flask was used in the first experiment. In the second experiment, 1% by weight of glucose, sucrose or maltose (Difco Laboratories, Detroit, Mich.) which had been filter-sterilized was added aseptically to each flask containing sterile NAK medium.

The gaseous environments of the flasks containing the medium were stabilized by supplying the flasks with appropriate gas mixtures and incubating in the shaker-water bath for 2 hr at 37 C prior to inoculation. One-half milliliter (1×10^5 CFU per ml) of inoculum was pipetted into each flask.

Incubation

A gyratory water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, N. J) with space for nine flasks was used. Each 250-ml Erlenmyer flask was fitted with a #6 1/2 rubber stopper through which were inserted two glass tubings with Tygon plastic tubings (I. D. 1/4; O. D. 3/8) for inlet and outlet of gases. The gas mixture from the cylinder was supplied to each flask through Tygon tubings. In order to eliminate evaporation of

the culture medium, the gas mixtures were saturated with water vapor by passing through 37 C water before reaching the sample flasks.

The incoming gas mixtures were filtered through cotton plugs that were placed in the glass tubing of the rubber stoppers. The rubber stopper assemblies were sterilized in an autoclave. The outgoing gas mixtures were also filtered through cotton plugs and were disposed of through a laboratory hood. The flow of gas mixtures was controlled manually using the gas pressure gauge. The gas mixtures (Prepure) were provided by Liquid Air Inc., Portland, Oregon.

The inoculated flasks were incubated in the water-bath at 37 C at an approximate shaking speed of 250 rpm/min time.

The sampling hours were 3, 5, 7, 11 and 16 for the cultures in air and 7, 11, 16, 20 and 24 for the cultures grown in 5% and 20% oxygen in nitrogen.

Colony forming units

Colony forming units (CFU) were determined by the pour plate method using plate count agar (Difco, Detroit, MI). Bacto-Peptone (Difco, Detroit, MI) at a 0.1% concentration in distilled water was used as a diluent. The plates were incubated for 24 hr at 37 C before being counted.

Dry weight

For the first experiment only, a 25-30 ml measured portion of the culture was centrifuged at 7,000 x g for 15 min. The sedimented cells were transferred into tared aluminum weighing dishes using 6 ml of physiological saline and dried for 24 hr at 85 C before being weighed.

Dissolved oxygen

An oxygen meter (Model 54, Yellow Springs Instrument Co., Yellow Springs, Ohio) was used to measure the dissolved oxygen in the culture media. The oxygen meter was calibrated by placing the probe in water-saturated air of known temperature and then adjusting the instrument to the ppm value found in an oxygen solubility table. The Clark type membrane-covered polarographic probe was immersed into the flask which was agitated slightly during the reading to prevent oxygen depletion around the cathode of the probe.

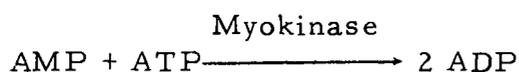
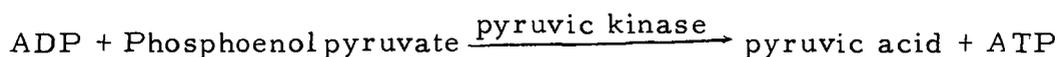
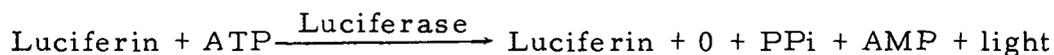
pH

A Beckman pH meter with a combination electrode was used for the measurement of pH.

Determination of adenosine phosphates

An ATP assay method utilizing luciferin-luciferase of the firefly bioluminescence system was used for the analysis of AMP,

ADP and ATP concentrations in the first experiment. This method is based on the following biochemical reactions:



Extraction. The extraction method of Atkinson (Chapman et al., 1971) was used with modifications based on preliminary studies. A 2-ml portion of the bacterial culture was added into an ice-cold test tube containing 0.4 ml of 35% HClO_4 . After standing in an ice-water bath for 15 min, the extract was neutralized with 1 ml of 2.6 N KOH. The denatured protein was removed by centrifugation, and the extract was immediately frozen. Due to the sampling hours in the experiment, the extracts had to be frozen over-night. Large variations were observed in the assay values of each adenosine phosphate fraction during a preliminary study. Several modifications were attempted unsuccessfully. Hot ethanol extraction, boiling the acid-culture mixture and ultrasonic vibration during acidification did not result in any apparent decrease in the variation or increase in average values.

Davidson and Fynn (1974) observed the presence of an ATP-degrading enzyme in cell-free extracts of Bacillus brevis.

The enzyme which was identified as an alkaline phosphatase, was found to be stable to perchloric acid precipitation and neutralization with potassium hydroxide resulting in an interference with the assay. The phosphatase activity was observed to be completely eliminated by removing the acid precipitate by centrifugation prior to neutralization. This observation was also confirmed by Atkinson (personal communication). Atkinson has also recommended the use of a KOH-KHCO₃ buffer for neutralization instead of KOH alone. A decrease in variability of assay values was observed in this experiment with the introduction of a centrifugation step prior to neutralization and the use of the buffer for neutralization.

The extraction method used in the study follows. Two ml of the culture were quickly removed from the flask and pipetted into a test tube containing 0.4 ml of ice-cold 35% perchloric acid. The mixture was vigorously agitated with a Vortex mixer for 30 sec and was allowed to stand for 15 min in an ice bath with periodic mixing with the Vortex mixer. The mixture was centrifuged at 7,000 x g for 10 min. A 1.6 ml portion of the supernatant was removed and neutralized with 0.52 ml of KOH (17% by weight)-KHCO₃ (5.8% by weight) to pH 7.0. Indicator paper (Dual-Tint, J. T. Baker Chemical Co., Phillipsburg, N. J.) was used to determine the desired pH. The suspension was thoroughly mixed with the Vortex mixer and left for 15 min in an ice bath for completion of the precipitation before being

centrifuged 10 min at 7,000 x g. The supernatant was transferred with a syringe, immediately frozen and stored no longer than 24 hr before the assay.

Sample dilution. Following the extraction of the adenosine phosphates from the cells, the method of Ching and Ching (1972) with a modification in dilution of extracts was used for the assay of ATP, ADP and AMP fractions. On the day of assay the frozen samples were thawed by immersing the tubes into a 30 C water bath. A 0.5 ml portion of the extract was diluted to 3 ml with 2.0 ml glass-distilled water and 0.5 ml of HEPES (Sigma Chemical Co., Saint Louis, Mo.) buffer (0.025 M, pH 7.5) containing 0.025 M Mg-acetate. For early hour samples (0-7 hr) 1.5 ml of the extract was diluted with 1.5 ml of HEPES buffer.

Preparation of reaction mixtures. A set of 3 tubes for each adenosine phosphate fraction was prepared marking the tubes as O, P, and M. The reaction mixtures (RM) were prepared according to the following table. Firefly lantern, pyruvate kinase and myokinase were from Sigma Chemical Co. (St. Louis, Mo.) Phosphoenol pyruvate was from Calbiochem (Los Angeles, Cal.).

Table 1. Reaction mixtures for adenosine phosphate assay

RM	Assay for	Buffer ¹	Phosphoenol pyruvate	Pyruvate kinase	Dialyzed myokinase
		ml	mg	μl	μl
O	ATP	1.0	0	0	0
P	ATP + ADP	1.0	4	40	0
M	ATP + ADP + AMP	1.0	4	40	80

¹HEPES-Mg. 0.025 M, pH 7.5

Incubation of the extracts with reaction mixture. A 100 μl portion of RM was pipetted into each tube (O, M, and P). The tubes were incubated for 15 min in a 30 C gyratory water bath and immediately cooled in an ice bath.

Assay of adenosine phosphates. An Aminco-Chem-Glow photometer (American Instrument Co., Silver Springs, Md.) was the instrument used. An ATP standard solution of 10^{-3} M in 0.025 M HEPES-Mg. pH 7.5 was prepared and diluted to 2.5×10^{-5} , 2.5×10^{-6} , and 2.5×10^{-7} with the buffer. The standards were kept in an ice bath.

A set of four reaction cells was prepared for each sample. Using a 1.0 ml syringe, 200 μl of the incubated mixture was transferred to each cell. A 10 μl portion of ATP standard was added to

two of the four cells. The instant peak height of the light emission was read on the photometer after adding 100 μ l of the firefly enzyme preparation.

Calculation. ATP in the reaction mixture was calculated as follows:

$$\text{ATP (nmole/ml)} = \frac{(\text{ave. } I_0)}{(\text{ave. } I^+) - (\text{ave. } I_0)} \times \frac{0.001 \times 25}{.021} \times \text{Dilution factor}$$

Dilution factor

$$10.494 \text{ for 6X dilution } \left(\frac{1.2}{1} \times \frac{1.06}{0.8} \times \frac{3}{0.5} \times 1.1 \right)$$

$$3.498 \text{ for 2X dilution } \left(\frac{1.2}{1} \times \frac{1.06}{0.8} \times \frac{3}{1.5} \times 1.1 \right)$$

ATP in O tube = ATP in extract

ATP in P tube = ATP + ADP in extract

ATP in M tube = ATP + ADP + AMP in extract

Therefore:

P - O = ADP in extract

M - P = AMP in extract

Energy charge was calculated as:

$$\text{E. C.} = \frac{(\text{ATP}) + \frac{1}{2} (\text{ADP})}{(\text{ATP}) + (\text{ADP}) + (\text{AMP})}$$

Enterotoxin assay

Approximately 8 ml of the sample were heat treated in a 50 C water bath for 10 min and centrifuged. The supernatant was

refrigerated until the time of analysis. Serological assays using gel-diffusion agar precipitation methods were used for detection and quantitation of enterotoxins.

Detection of enterotoxin. Crowle's microslide technique, as described by Casman et al. (1969) was used to detect the enterotoxins.

The purified enterotoxins and antisera for C used for these assays were obtained from M. S. Bergdoll (Food Research Institute, University of Wisconsin). Antisera for enterotoxins A and B were produced in this laboratory. The sample was placed in the side wells and an appropriate dilution of control enterotoxin in the top and bottom wells as the reference. An appropriate dilution of antiserum was placed in the center well. The slides were incubated for 24 hr at 37 C or for 48 hr at room temperature. After removing the template, the slides were stained with 0.1% Thiazine Red R dye (Crowle, 1958; Casman et al. (1969) for scanning of precipitin lines. The negative samples were concentrated approximately 15 times with Aquacide (Calbiochem, Los Angeles, Cal.) for 24 to 48 hr at 4 C in the refrigerator. The samples were centrifuged and dialyzed against the culture medium (NAK broth), and reset on microslides. The detection limit for this method was 0.2 μ g per ml.

Quantitation of enterotoxins. The Oudin method (Hall, Angelotti, and Lewis, 1963) was used for quantitation of enterotoxins in the samples in which enterotoxins had been detected by

the microslide technique. The sample was placed on top of the prepared agar column. The tubes were incubated at 30 C in a water bath. The migration of the precipitin band was recorded three times during a three day period, using a cathetometer with a short-focus telescope (Gaertner Scientific Corporation, Chicago, Ill.). The slope of the reading was then compared to a standard curve for estimation of the amount of enterotoxin. The detection limit for this method was 4 $\mu\text{g}/\text{ml}$.

Statistical analysis

The logarithmic transformation was made for the number of colony forming units and the weight of enterotoxins. For the first experiment 3 x 3 factorial analysis was done on the effects of treatment, sampling times and replication. For the second experiment, 3 x 4 factorial analysis was made on the effects of sugar, treatment, sampling time and replication.

RESULTS AND DISCUSSION

In evaluating the effect of atmosphere on the culture of S. aureus, the attributes measured, such as CFU, DO, and pH are all reflections of the total metabolism. For clarity, however, each will first be discussed individually before the total composite is summarized. The discussion is presented first for the main experiment, the simple 4% NAK growth medium.

Growth

Colony forming units (CFU) and dry weight were used for the measurement of growth. Both in CFU and dry weight, growth was fastest in air for all three strains, followed by 20% and 5% oxygen in nitrogen (Figure 1 and Table 2).

Although each strain reached a CFU of approximately 1×10^{10} cells per ml at the stationary phase of growth under each of the three atmospheric conditions, the duration of the growth phase varied with each atmosphere. The effect of the atmosphere was greatest at the earlier sampling hours; the interaction of time with treatment (atmosphere) was significant at the 0.01 level (Table 3). The lag phase was more prolonged in 5% and 20% oxygen in nitrogen than it was in air. In air, the exponential phase ended between 7 and 9 hr of growth in all strains; in 20% oxygen in nitrogen, between 11 and 16 hr; and

FIG. 1
GROWTH CURVE OF THREE STRAINS OF *S. AUREUS*
DURING GROWTH IN THREE ATMOSPHERES.

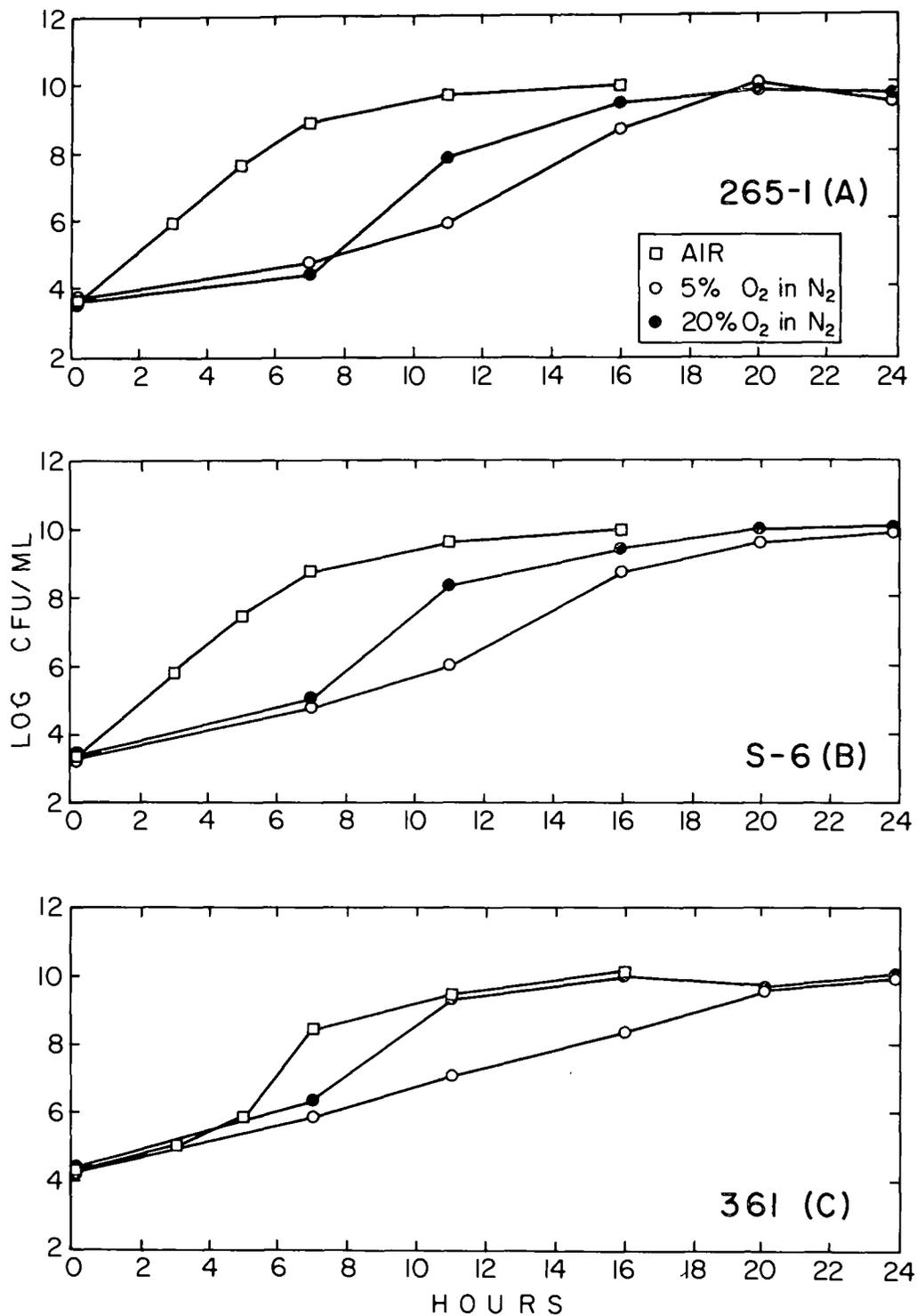


Table 2. Colony forming units (CFU) and dry weight for three strains of *S. aureus* during growth under three different atmospheres. ^{a, b}

Sampling Time hr.	265-1						S-6						361					
	Air		5% O ₂ in N ₂		20% O ₂ in N ₂		Air		5% O ₂ in N ₂		20% O ₂ in N ₂		Air		5% O ₂ in N ₂		20% O ₂ in N ₂	
	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)	CFU (log)	Dry Weight (µg/ml)
0	3.67		3.67		3.67		3.33		3.33		3.33		4.35		4.35		4.35	
3	5.93	< 30					5.76	< 30					5.08	< 30				
5	7.62	< 30					7.43	32 (9) ^c					5.86	< 30				
7	8.83	300 (120)	4.71	< 30	4.43	< 30	8.69	370 (99)	4.77	< 30	5.01	< 30	8.43	390 (120)	5.89	< 30	6.35	< 30
11	9.68	530 (190)	5.91	< 30	7.88	< 30	9.57	1100 (320)	6.01	< 30	6.63	100 (150)	9.44	500 (250)	7.08	59 (53)	9.49	650 (18)
16	9.92	700 (120)	8.67	370 (350)	9.49	670 (140)	9.87	1300 (300)	8.69	700 (250)	9.43	1200 (150)	10.07	1100 (370)	8.35	300 (310)	10.08	890 (820)
20			9.96	830 (120)	9.76	840 (77)			9.53	980 (340)	9.89	1200 (120)			9.79	980 (350)	9.57	960 (450)
24			9.52	1200 (87)	9.92	970 (200)			9.89	1370 (250)	10.01	1300 (210)			10.42	1460 (300)	10.14	2400 (860)

^a Blank space indicates that sampling was not scheduled.

^b Average of three replications.

^c Standard deviation.

Table 3. Mean values and significance of variance of colony forming units, dissolved oxygen, pH, energy charge and enterotoxin production in cultures of three strains of *S. aureus* during growth in three atmospheres.

Strain	Treatment			F ^a	Sampling Time (hr)			
	Air	5% in O ₂ in N ₂	20% O ₂ in N ₂		7	11	16	F
CFU (log/ml)								
265-1	9.74	6.30	7.26	19.89**	5.99	7.82	9.36	13.79**
	Time x Treatment			12.53**				
S-6	9.38	6.49	9.31	53.93**	6.16	7.40	9.34	57.36**
	Time x Treatment			6.30				
361	9.31	7.11	8.64	8.57**	6.89	8.67	9.68	11.91**
	Time x Treatment			0.94				
DO (ppm)								
265-1	0.4	1.2	3.6	62.69**	2.7	1.7	0.7	33.80**
	Time x Treatment			21.51**				
S-6	0.7	1.6	2.1	115.64**	3.4	2.7	0.4	76.47**
	Time x Treatment			39.43**				
361	0.5	1.5	2.9	5.39**	2.7	0.5	0.7	19.15**
	Time x Treatment			14.61**				
pH								
265-1	7.4	6.9	6.9	4.74*	6.8	7.1	7.4	4.04*
	Time x Treatment			2.18				
S-6	7.4	6.7	6.9	74.00**	6.7	7.0	7.3	52.19**
	Time x Treatment			27.12**				
361	7.3	6.9	7.1	4.09*	6.7	7.0	7.5	15.10**
	Time x Treatment			4.39*				
Energy Charge								
265-1								
	Time x Treatment							
S-6	0.49	0.09	0.32	15.71**	0.18	0.38	0.37	4.19
	Time x Treatment			0.05				
361	0.59	0.32	0.41	11.05**	0.34	0.47	0.50	4.11
	Time x Treatment			4.17*				
Enterotoxin (ug/ml)								
265-1	111	25	44	11.84**	19	69	189	12.65**
	Time x Treatment			2.18				
S-6	2050	771	2710	6.37*	20	21	3443	5.09*
	Time x Treatment			2.79				
361	923	593	4120	6.75**	27	1243	4367	28.30**
	Time x Treatment			2.14				

a Statistical significance in strain by * for 0.05 level; **, 0.01 level.

in 5% oxygen in nitrogen, at 20 hr of growth.

A strain difference in growth response to different atmospheric conditions was observed. The growth curves for strains 265-1 and S-6 in the air were very similar and the growth responses to 5% and 20% oxygen level in nitrogen were similar; however, strain 361 had a slightly different growth curve in the air and it also differed in growth response to the other gas mixtures from the other two strains. The lag phase was longer and the exponential phase was steeper in strain 361 than in strains 265-1 and S-6 in the air. The growth of strain 361 was least affected among the strains by 20% oxygen in nitrogen. The growth curves of strain 361 in the air and in 20% oxygen were almost identical at all hours except between 5-11 hr of growth when the cells in the air had an earlier exponential phase.

Barber and Diebel (1972) conducted a study with sausage in which the effect of several levels of oxygen on staphylococcal growth was investigated. When a strain producing enterotoxin A was inoculated into sausage and incubated at 37 C under 5%, 10% and 15% oxygen in air, it was observed that the cells in 5% oxygen took 2-3 days to reach cell numbers comparable to those reached in 24 hr by the cells grown in air. However, in the present experiment, the three strains in 5% oxygen in nitrogen attained in 24 hr the maximal CFU achieved in air. The discrepancy in the growth rates of the cells in the two experiments may be due to the difference in medium used.

The concentration of the salts in the sausage might have enhanced the effect of 5% oxygen, resulting in a greater inhibition of growth.

Due to a great variation in replicate values, it would not be possible to derive any positive conclusion from the dry weight data. However, the response was similar to that observed in CFU. Increase in dry weight was fastest in air, followed by 20% and 5% oxygen in nitrogen in all the strains. The slight effect of 20% oxygen in nitrogen on strain 361 shown in CFU was also observed in dry weight (Table 2).

From these observations, it could be concluded that growth of S. aureus is delayed in 20% and 5% oxygen in nitrogen, with a greater delay at the lower concentration. Although the efficacy of growth in aerobiosis is generally attributed to the availability of oxygen, it was observed in this study that another component besides oxygen concentration affected growth. Thus, it may not be justifiable to regard a 20% oxygen atmosphere as air in growth studies as has been done in some investigations. It has been reported that in the complete absence of carbon dioxide, staphylococci do not grow (Angelotti, 1969; Elek, 1959). More abundant growth of S. aureus in air than in 20% oxygen may be due to the presence of carbon dioxide (0.03%) in the air.

Dissolved oxygen concentration

Concentrations of dissolved oxygen (DO) in the culture of the

FIG. 2
 DISSOLVED OXYGEN CONCENTRATION IN CULTURES OF
 THREE STRAINS OF S. AUREUS DURING GROWTH IN
 THREE ATMOSPHERES.

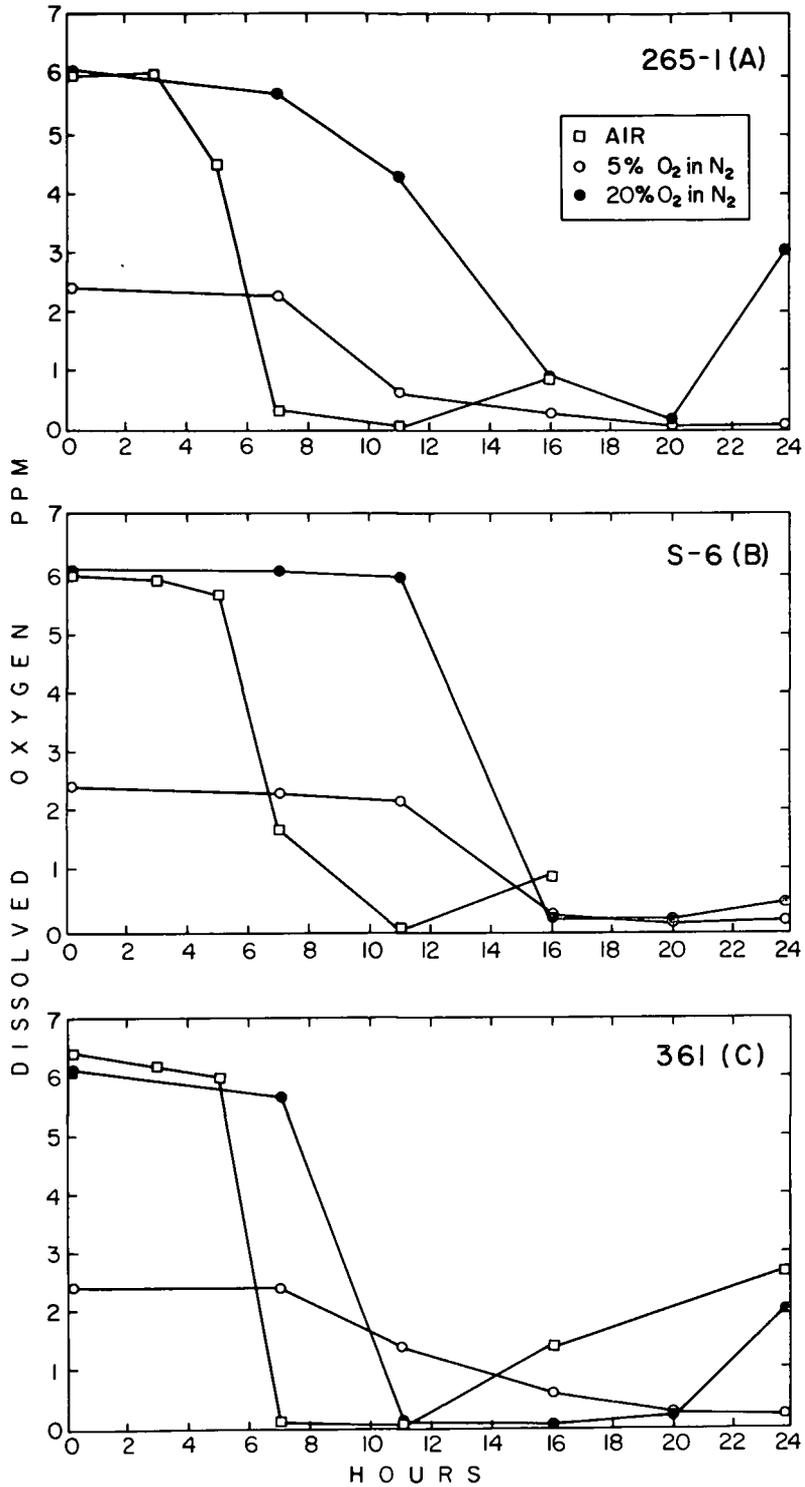


Table 4. Dissolved oxygen (ppm) and pH in cultures of three strains of *S. aureus* during growth under three different atmospheres. a, b

Dissolved Oxygen (DO)									
Sampling Time hr	265-1			S-6			361		
	Air	5% O ₂ in N ₂	20% O ₂ in N ₂	Air	5% O ₂ in N ₂	20% O ₂ in N ₂	Air	5% O ₂ in N ₂	20% O ₂ in N ₂
0	6.4	2.4	6.4	6.4	2.4	6.4	6.4	2.4	6.4
3	6.0			5.9			6.2		
5	4.5			5.7			6.0		
7	0.3	2.3	5.7	1.7	2.3	6.1	6.1	2.4	5.7
11	0.0	0.9	4.3	0.0	2.2	6.0	0.0	1.4	0.1
16	0.9	0.3	0.9	0.9	0.2	0.2	1.4	0.6	0.0
20		0.1	0.2		0.1	0.2		0.2	0.2
24		0.1	3.1		0.2	0.5	2.7	0.2	1.0

pH									
Sampling Time hr	265-1			S-6			361		
	Air	5% O ₂ in N ₂	20% O ₂ in N ₂	Air	5% O ₂ in N ₂	20% O ₂ in N ₂	Air	5% O ₂ in N ₂	20% O ₂ in N ₂
0	6.8	6.8	6.8	6.7	6.7	6.7	6.8	6.8	6.8
3	6.7			6.7			6.8		
5	6.8			6.7			6.7		
7	6.7	6.8	6.8	6.7	6.7	6.8	6.6	6.8	6.8
11	7.5	7.0	6.8	7.5	6.7	6.7	7.0	6.9	7.1
16	7.9	6.8	7.0	8.0	6.7	7.0	8.2	7.0	7.4
20		6.9	8.5		6.9	8.0		7.2	7.6
24		7.5	7.8		7.3	8.3	8.3	8.7	8.0

a Blank space indicates that sampling was not scheduled.

b Average of three replications.

three strains growing under three atmospheric conditions are shown in Figure 2 and Table 4. The depletion of DO in the culture medium closely reflected the growth phases of the cells in all strains under these environments. During the lag phase, the DO concentration changed very little. It was depleted rapidly during the exponential phase when the cellular oxygen demand was great and oxygen was metabolized as rapidly as it dissolved in the medium. The concentration of DO started to increase at the beginning of the stationary phase when the cellular energy need decreased in all three strains.

There was a slight strain difference in the rate of depletion of DO in the culture media. The depletion of the DO in strain 265-1 grown in air was slightly faster than that either in strain S-6 or in strain 361, although the growth curve in air was almost identical in strains 265-1 and S-6. A negligible effect of 20% oxygen in nitrogen on growth of strain 361 was also reflected in the depletion pattern of DO in this strain. The depletion of DO concentration under incubation in air had occurred in all three strains by 7-8 hr.

As for CFU, the effect was greatest at the earlier sampling hours; the interaction of treatment and time was highly significant (Table 3).

pH

No apparent strain differences were found in the level of pH in cultures of the three strains (Table 4). The starting pH of the

culture medium was 6.8. During growth in air, each strain reached a pH of about 7 at 11 hr and about 8 at 16 hr. During growth in 5% and 20% oxygen in nitrogen, the increase in pH was slightly slower than it was during growth in air; all three strains reached a pH of 7 between 11 and 16 hr and that of 8 between 20 and 24 hr. The analysis of variance showed that the effect of the three atmospheres and also that of sampling time were significant in all strains. The interaction of treatment-time was significant in strains S-6 and 361, but not in strain 265-1 (Table 3).

Adenosine phosphate and energy charge

The adenosine phosphate content and the adenylate energy charge during growth in air of S. aureus strains 265-1, S-6 and 361 are shown in Figures 3, 4, and 5 and Tables 5, 6, and 7. The value of energy charge changed during the early period of growth in all the strains. The difference was tested statistically only for strains S-6 and 361 at the 7, 11 and 16 hr periods when all gaseous atmospheres were sampled; at these hours, the effect of time was not significant (Table 3). The energy charge levels were somewhat lower than in reported studies in E. coli. Chapman et al. (1971) reported that growth of E. coli can occur only at energy charge values above 0.8, that viability is maintained at values between 0.8 and 0.5 and that cells die at values below 0.5. Dietzler et al. (1974) observed that energy

FIG. 3

ENERGY CHARGE, ENTEROTOXIN PRODUCTION AND CHANGES IN ADENOSINE PHOSPHATES AND DISSOLVED OXYGEN DURING GROWTH OF S. AUREUS STRAIN 265-1 IN AIR.

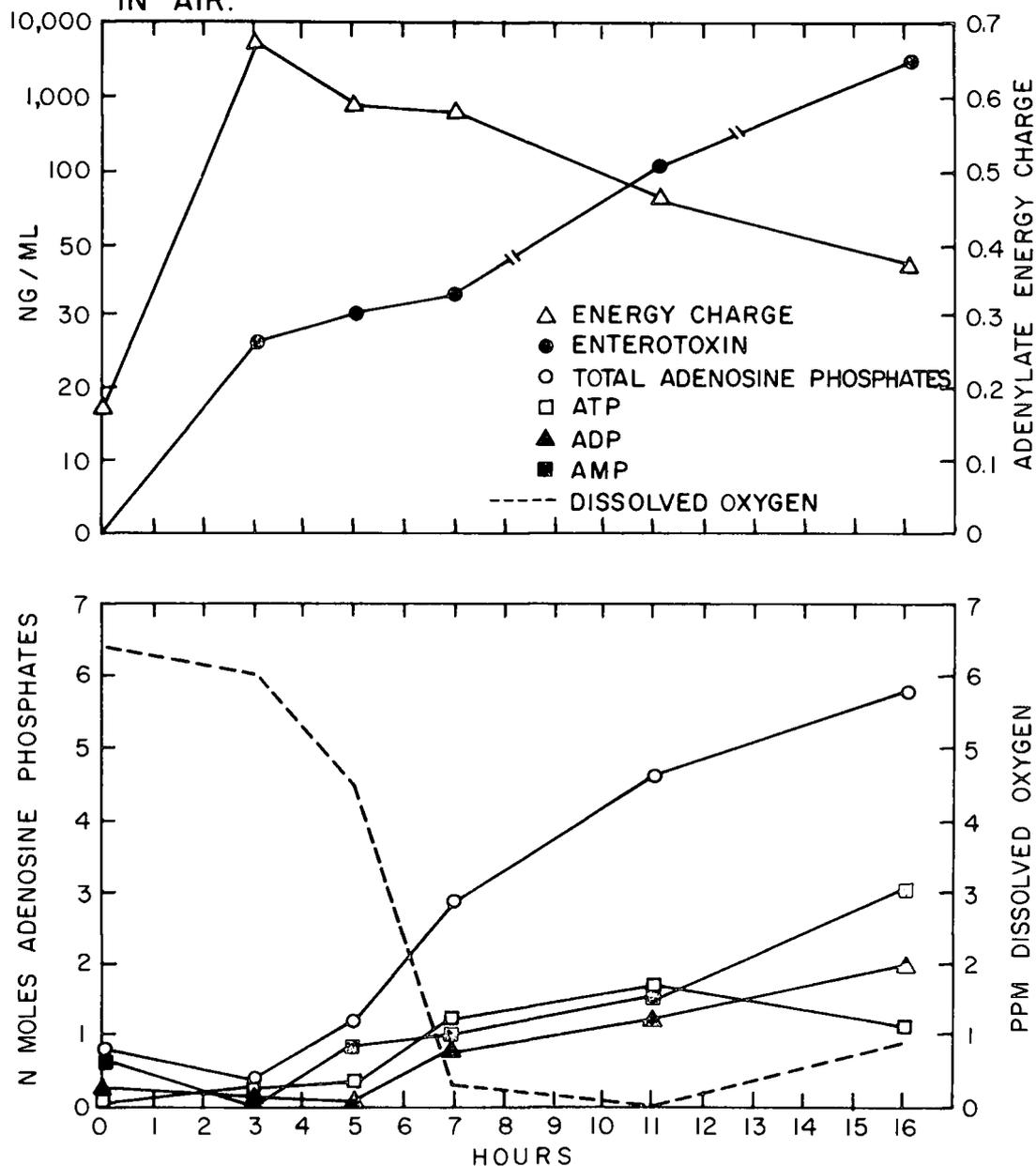


FIG. 4
 ENERGY CHARGE, ENTEROTOXIN PRODUCTION AND
 CHANGES IN ADENOSINE PHOSPHATE AND DISSOLVED
 OXYGEN DURING GROWTH OF S. AUREUS STRAIN S-6
 IN AIR.

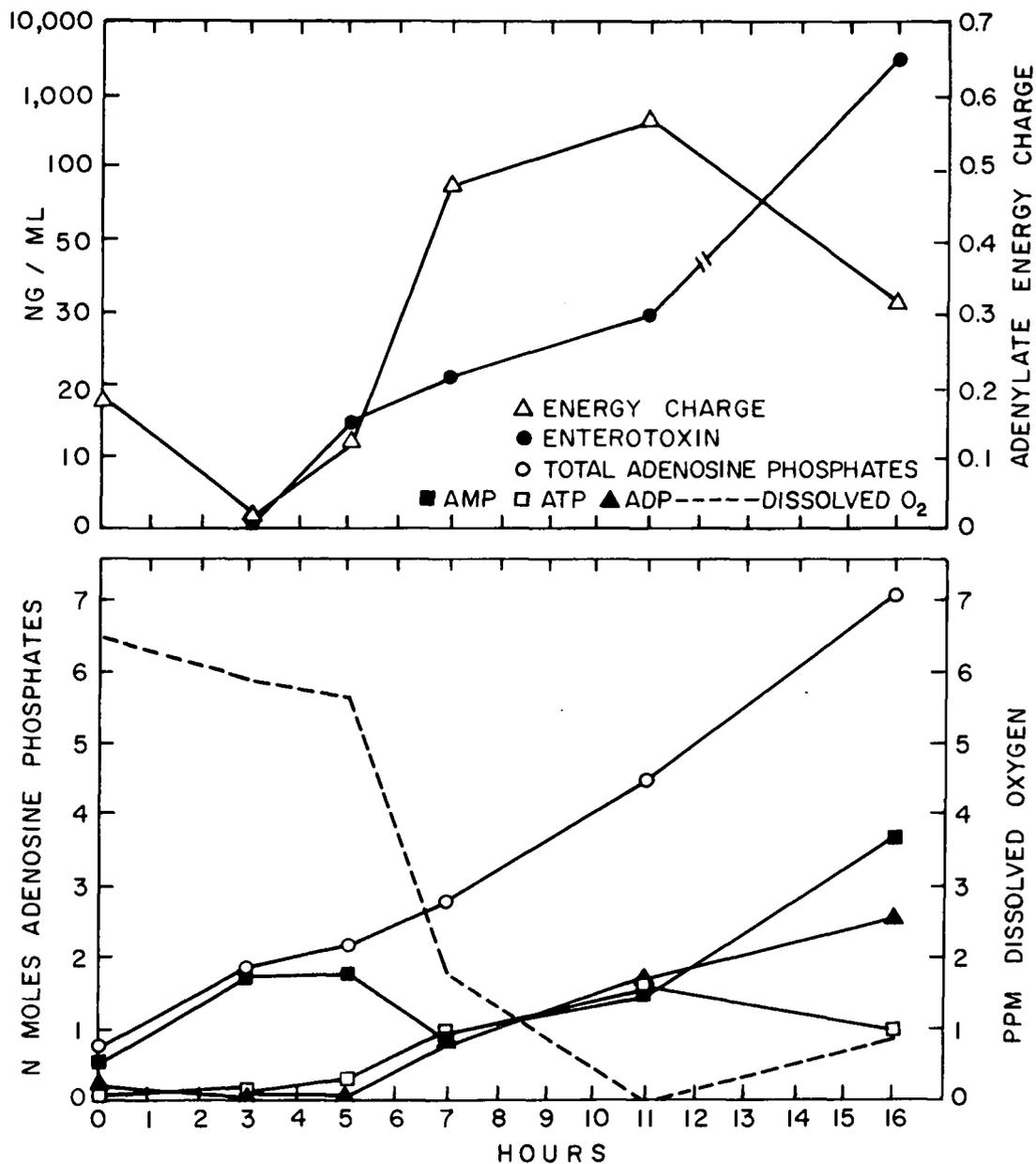


FIG. 5
 ENERGY CHARGE, ENTEROTOXIN PRODUCTION AND
 CHANGES IN ADENOSINE PHOSPHATE AND DISSOLVED
 OXYGEN DURING GROWTH OF S. AUREUS STRAIN 361
 IN AIR.

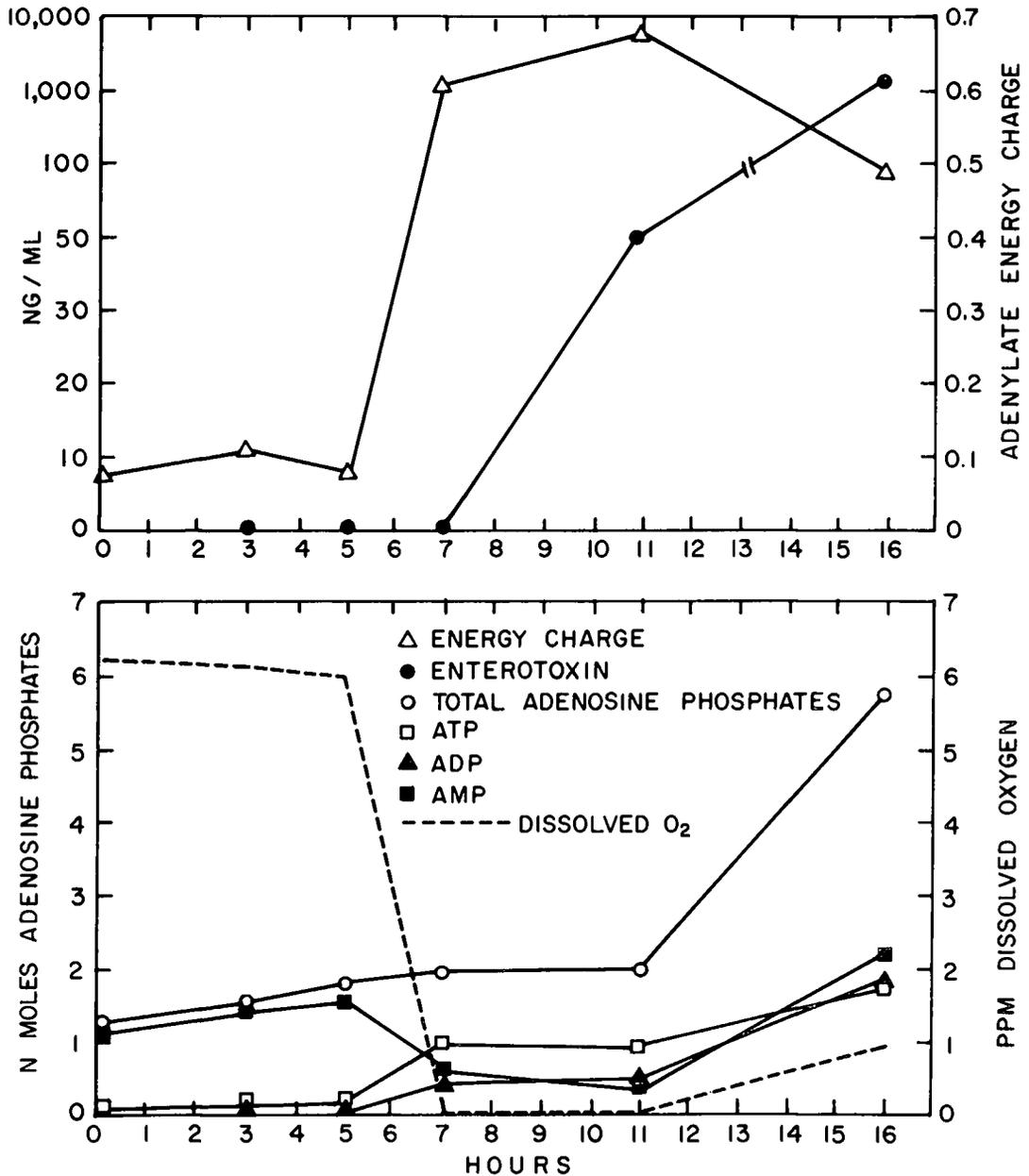


Table 5. Adenosine phosphate concentration in n moles/ml and energy charge with standard deviation in three strains of *S. aureus* during growth in air.^a

Sampling Time hr	265-1				
	ATP	ADP	AMP	Total	E. C. ^b
0	.019 (.009) ^c	.193 (.060)	.642 (.467)	.835 (.496)	.17 (.07)
3	.121 (.060)	.167 (.056)	0 (0)	.266 (.045)	.68 (.09)
5	.371 (.306)	.115 (.091)	.179 (.010)	.473 (.039)	.59 (.08)
7	1.247 (.489)	.821 (.301)	.822 (.575)	2.889 (.405)	.58 (.11)
11	1.538 (.409)	1.205 (.373)	1.599 (.242)	4.577 (.405)	.46 (.08)
16	1.105 (.035)	1.906 (.733)	3.094 (.606)	5.706 (1.408)	.37 (.02)
S-6					
0	.018 (.003)	.203 (.041)	.898 (.353)	1.119 (.146)	.11 (.01)
3	.027 (.010)	.015 (.009)	1.843 (.040)	1.897 (.038)	.02 (.00)
5	.240 (.096)	.087 (.046)	1.869 (.028)	2.233 (.086)	.13 (.05)
7	.927 (.371)	.929 (.150)	.914 (.718)	2.770 (.497)	.49 (.15)
11	1.619 (.334)	1.867 (.574)	1.683 (.499)	4.546 (.938)	.57 (.05)
16	1.025 (.315)	2.609 (.611)	3.765 (1.930)	7.091 (.684)	.32 (.15)
361					
0	.066 (.006)	.030 (.016)	1.183 (.567)	1.261 (.565)	.08 (.04)
3	.070 (.023)	.015 (.005)	1.537 (.494)	1.586 (.514)	.11 (.09)
5	.079 (.015)	.029 (.010)	1.515 (.127)	1.759 (.041)	.07 (.02)
7	.988 (.265)	.463 (.047)	.558 (.086)	1.952 (.434)	.60 (.00)
11	.805 (.442)	.450 (.351)	.488 (.175)	1.937 (1.247)	.68 (.02)
16	1.724 (.677)	1.739 (.356)	2.285 (.305)	5.736 (.305)	.49 (.12)

a Average of three replications.

b $E. C. = \frac{(ATP) + (\frac{1}{2} ADP)}{(ATP) + (ADP) + (AMP)}$

c Standard deviation.

Table 6. Adenosine phosphate concentration in n moles/ml and energy charge with standard deviation in two strains of *S. aureus* during growth in 20% O₂ in N₂.^a

Sampling Time hr	S-6				E. C. ^b
	ATP	ADP	AMP	Total	
7	.023 ^c (.018) ^c	.044 (.015)	1.675 (.590)	1.751 (.558)	.04 (.02)
11	.258 (.026)	.244 (.148)	1.366 (.338)	1.867 (.236)	.19 (.23)
16	1.135 (.494)	1.214 (.536)	1.831 (1.133)	4.180 (1.346)	.44 (.17)
361					
7	.390 (.129)	.675 (.079)	.675 (.632)	1.736 (.358)	.39 (.04)
11	.787 (.525)	.345 (.013)	.481 (.168)	1.610 (1.596)	.66 (.00)
16	1.035 (.357)	1.288 (.529)	1.539 (.060)	4.093 (.439)	.43 (.07)

a Average of three replications.

b E. C. = $\frac{(ATP) + (1/2 ADP)}{(ATP) + (ADP) + (AMP)}$

c Standard deviation.

Table 7. Adenosine phosphate concentration in n moles/ml and energy charge with standard deviation in two strains of *S. aureus* during growth in 5% O₂ in N₂.^a

Sampling Time hr	S-6				
	ATP	ADP	AMP	Total	E. C. ^b
7	.015 (.002) ^c	.023 (.006)	1.517 (.386)	1.600 (.334)	.02 (.00)
11	.078 (.054)	.066 (.047)	1.636 (.162)	1.780 (.169)	.07 (.01)
16	.945 (.234)	.986 (.158)	1.209 (.299)	2.507 (.104)	.52 (.06)
361					
7	.186 (.170)	.015 (.009)	1.717 (.444)	1.796 (.433)	.04 (.01)
11	.420 (.047)	.133 (.077)	.680 (.541)	1.233 (1.165)	.31 (.19)
16	.705 (.015)	.119 (.081)	.180 (.070)	.776 (.646)	.60 (.15)

a Average of three replications.

$$b \text{ E. C.} = \frac{(ATP) + (1/2 ADP)}{(ATP) + (ADP) + (AMP)}$$

c Standard deviation.

charge was strongly poised at 0.86-0.89 in E. coli even when the total adenylate concentrations were lowered by manipulating the growth conditions.

There was a definite strain difference in the pattern of change in energy charge during growth of staphylococci, especially during the lag and exponential phases. In strain 265-1, the energy charge increased very rapidly and had reached 0.68 at 3 hr of growth followed by a gradual decrease reaching 0.37 at 16 hr. The energy charge in strains S-6 and 361 reached an equivalent peak much later (at about 8 hr). In strain S-6 the energy charge decreased to 0.02 during the early hours of growth; in strain 361, it remained at around 0.1 during this period and started to increase rapidly. In both strains, the energy charge started to decrease at the early part of the stationary phase.

Reported studies concerning adenosine phosphate concentration and the adenylate energy charge in microorganisms are rather limited at this time and no data have been published on S. aureus. Even among the reported values of adenylate energy charge, there is a great discrepancy. The energy charge values ranged from 0.20 in Saccharomyces cerevisiae to 0.90 in E. coli in a tabulation calculated from adenylate nucleotide levels in 20 microorganisms (Chapman et al., 1971). Variation in energy charge value has been attributed mainly to the differences in the analytical methods employed. It

cannot be ruled out that the variations may also be due to species differences or to the growth phase at which the analyses were made.

The pattern of change in the pool size of total adenosine phosphate was also different among the strains at the early part of growth (Figures 3, 4 and 5). In strain 265-1, the pool size started to increase rapidly about 3 hr after an initial decrease. There was no initial decrease of the pool size in strains S-6 and 361; it kept increasing steadily but the rate of increase was much faster in strain S-6 than it was in strain 361. The pattern of change in the concentrations of each nucleotide also varied among the strains during the lag and exponential phases; however, in the later stages the changes in the three nucleotides were similar in all the strains. Standard deviations are given in Tables 5, 6 and 7. Although the variation was occasionally large, when data were plotted for each replication, the trend was uniform.

The adenosine phosphate content and the adenylate energy charge during growth in 5% and 20% oxygen were measured only in strains S-6 and 361 and the sampling was done at three periods (7, 11 and 16 hr of growth). The data are shown in Tables 6 and 7. The analysis of variance showed that the effect of the three atmospheres on energy charge was significant at the 0.01 level in both strains. However, the treatment-time interaction was significant for strain 361 (Table 3). In both 5% and 20% oxygen in nitrogen as

Table 8. Enterotoxin ($\mu\text{g/ml}$) production of three strains of *S. aureus* under three different atmospheres. ^{a, b}

Sampling Time hr	265-1			S-6			361		
	Air	5% O ₂ in N ₂	20% O ₂ in N ₂	Air	5% O ₂ in N ₂	20% O ₂ in N ₂	Air	5% O ₂ in N ₂	20% O ₂ in N ₂
3	.03			0			0		
5	.03			.02			0		
7	.03	.01	.02	.02	.02	.02	0	.02	.60
11	.15	.03	.03	.03	.02	.01	.04	.09	3.6
16	.50	.04	.09	6.1	2.2	2.0	2.7	1.7	8.7
20		.06	.40		12	21		16	22
24		.73	.40		17	24		25	32

a Blank space indicates that sampling was not scheduled.

b Average of three replications.

compared to air, energy charge increased after a longer lag period.

Enterotoxin production

Enterotoxin production by the three strains during growth under three different atmospheric conditions is shown in Table 8. As expected, time had a significant effect (Table 3). A difference in the time sequence of enterotoxin production in air was observed among strains. Enterotoxin A by strain 265-1 was detected at 3 hr of growth whereas enterotoxin B by strain S-6 and enterotoxin C by strain 361 were detected at 5 hr and 11 hr, respectively. The time before production of detectable amounts of enterotoxins A and B observed in this study is in accordance with reports by Markus and Silverman (1968, 1970). They reported that enterotoxin A was produced during the exponential phase and enterotoxin B was produced during the later part of the exponential and the early part of the stationary phase. In this study a measureable amount of enterotoxin C production by strain 361 was first detected at the stationary phase. According to the postulation of Markus and Silverman (1968, 1970), enterotoxin C would be a secondary metabolite.

Strain differences were also observed in the amounts of enterotoxin produced as has been found by others. It has been known that enterotoxin B is produced in highest amount followed by enterotoxins C and A. In the present study, the production of enterotoxins were

measured only up to 16 hr of growth for A and B and 24 hr only for enterotoxin C. Therefore, it is not possible to compare the values found with reported values which were generally measured at 24 hr of growth. At 16 hr of growth in air, 6.1 μg per ml of enterotoxin B was produced by strain S-6; enterotoxin C produced by strain 361 was 2.7 μg per ml and enterotoxin A by strain 265-1 was 0.5 μg per ml. At 24 hr, strain 361 produced 20 μg per ml of enterotoxin C.

Strain differences of S. aureus in response to environmental conditions were also observed in enterotoxin production. The effect of the atmosphere on enterotoxin production was significant for the three strains (Table 3). In strains 264-1 and S-6, a similar response shown in growth was observed in enterotoxin production was faster and in greater amount in air than it was in either 5% or 20% oxygen in nitrogen. In 5% and 20% oxygen in nitrogen, enterotoxin A was detected at 7 hr of growth, whereas in air it was detected at 3 hr. The amount of enterotoxin A produced under both atmospheres was similar. In strain S-6, a detectable amount of enterotoxin B was first produced at 7 hr in 5% and 20% oxygen in nitrogen whereas in the air, it was at 5 hr. The amount of enterotoxin B produced in the three atmospheres was similar (Table 8).

The response of strain 361 in enterotoxin production to

environmental conditions was distinctively different from the other two strains. In this strain the atmosphere of 5% and 20% oxygen in nitrogen had a stimulatory effect on the production of enterotoxin C. In air, enterotoxin C was not detected until 11 hr of growth, whereas it was detected at 7 hr under the other two atmospheres. The amounts of enterotoxin C produced at 24 hr differed little: 20, 25 and 32 μg per ml in the air, 5% oxygen and 20% oxygen in nitrogen, respectively.

Composite of microbial growth and metabolism

Since the measures used to follow changes in the S. aureus cultures are interrelated, one should also look at the composite. As the growth rate increased, oxygen demand and energy charge increased. Subsequently, enterotoxin could be detected and pH decreased in the culture media incubated in air. There was some evidence for strain differences in that strain 361 had a lag in production of enterotoxin following the increase in energy charge and responded differently to the gaseous atmospheres employed. In the culture media of strains S-6 and 361 incubated in 5% and 20% oxygen in nitrogen, enterotoxins Band C respectively were detected at 7 hr of growth when the growth was still at lag phase and the energy charge values were low. In strain 361, the atmospheres of 5% and 20% oxygen in nitrogen had a stimulatory effect

on enterotoxin production.

Recently it has been suggested that the level of ATP might be a controlling factor in enterotoxin secretion (Carpenter and Silverman, 1974). In the present study, it was observed that the synthesis of enterotoxin accompanied a rapid increase in energy charge, regardless of the time sequences of production (Figures 3, 4 and 5). Enterotoxin A was detected in strain 265-1 at 3 hr of growth when the energy charge was at a peak; no enterotoxin was detected in the other two strains at this time. A detectable amount of enterotoxin B appeared at 5 hr in strain S-6 when the energy charge was increasing. (No enterotoxin A was detected at any time in cultures of strain S-6.) A dissimilar trend in relationship between the levels of energy charge and the production of enterotoxin was observed during the growth of strains 265-1 and S-6 in 5% and 20% oxygen in nitrogen (Tables 6, 7 and 8). In strain 361, however, there was a possible lag between the time of a rapid increase in energy charge and that of production of a detectable amount of enterotoxin C; the energy charge increased rapidly at 7 hr and enterotoxin was first detected at 11 hr in air.

The appearance of enterotoxin was also accompanied by a rapid decrease in dissolved oxygen tension in the cultures. It is apparent that the synthesis of enterotoxin occurred at higher energy charge conditions and that the enterotoxin production may be

controlled by the energy metabolism of the organism. In this study no apparent relationships were found between enterotoxin synthesis and the concentrations of each nucleotide during growth (Figures 3, 4 and 5). The concentrations of the nucleotides are related to numbers of cells as well as growth phase.

More data are needed before we could discern the precise relationship of energy metabolism during growth and enterotoxin production of S. aureus. Measuring the levels of ATP, ADP and AMP and the energy charge would be a valuable approach in probing into energy metabolism.

Effect of added sugars

Using strain 265-1, for the second experiment, the effect of sugars added to the 4% NAK medium on growth, DO, pH and enterotoxin production was investigated. Three sugars, glucose, maltose and sucrose, were each used at a 1% level. Sampling times were 3, 5 and 7 hr. The data are shown in Table 9 and Figure 6.

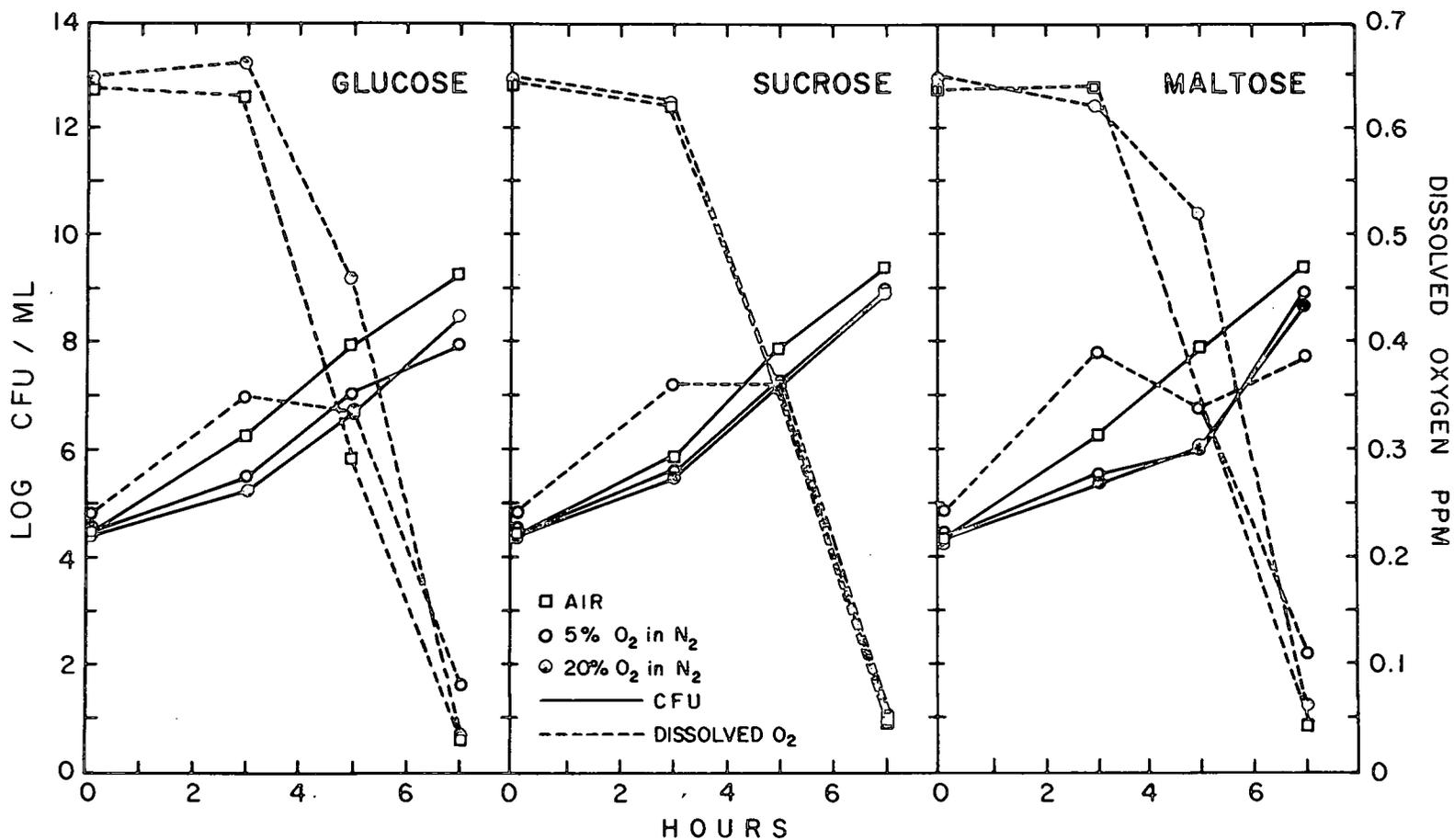
During growth in air, the addition of sugar had very little effect on CFU at 3 and 5 hr. In NAK medium without added sugar, CFU of strain 265-1 at 3 hr was 86×10^4 per ml; it was $70 \times 10^4 - 19 \times 10^5$ per ml when grown in the presence of three sugars. At 5 hr, the CFU in NAK culture was 42×10^6 per ml

Table 9. Colony forming units (CFU), dissolved oxygen (DO), pH and enterotoxin production of *S. aureus* strain 265-1 during growth in three atmospheres in medium with added sugars.

Atmosphere	Sampling Time hr	Sugars											
		Glucose				Sucrose				Maltose			
		CFU (log)	DO (ppm)	pH	Enterotoxin	CFU (log)	DO (ppm)	pH	Enterotoxin	CFU (log)	DO (ppm)	pH	Enterotoxin
Air	3	6.26	6.3	6.7	0/3 ^a	5.84	6.2	6.7	0/3	6.27	6.4	6.6	0/3
	5	7.91	2.9	6.6	0/3	7.82	3.6	6.7	0/3	7.89	3.6	6.5	0/3
	7	9.28	0.3	5.9	1/3	9.37	0.7	6.1	1/3	9.41	0.3	6.2	2/3
5% O ₂ in N ₂	3	5.43	3.5	6.6	0/3	5.60	3.6	6.7	0/3	5.51	3.9	6.7	0/3
	5	7.05	3.3	6.6	0/3	7.18	3.6	6.6	0/3	6.02	3.4	6.6	2/3
	7	8.00	1.8	6.1	2/3	8.90	0.4	6.2	2/3	8.94	1.1	6.2	2/3
20% O ₂ in N ₂	3	5.25	6.6	6.6	1/3	5.56	6.2	6.6	1/3	5.41	6.2	6.6	1/3
	5	6.71	4.6	6.6	1/3	7.21	3.4	6.6	1/3	6.07	5.2	6.6	1/3
	7	8.49	0.3	6.3	3/3	8.90	0.9	6.3	3/3	8.76	0.6	6.4	3/3

a Replications with enterotoxin production/total replications.

FIG. 6
 COLONY FORMING UNITS AND DISSOLVED OXYGEN CONCENTRATION
 OF *S. AUREUS* 265-1 CULTURE DURING GROWTH IN AIR, 5% O₂ IN N₂
 AND 20% O₂ IN N₂ WITH ADDED SUGARS.



and that in the medium with added sugars was 66×10^6 - 81×10^6 per ml. At the later hours, the cultures with added sugars had slightly higher CFU than did NAK medium alone. At 7 hr, the CFU in the simple medium was 68×10^7 per ml and it was 19×10^8 - 26×10^8 per ml when sugars were added. Jarvis and Lawrence (1975) reported that the specific growth rate of S. aureus strain S-6 grown in a fermenter in a synthetic medium increased rapidly when glucose was added. No significant difference was observed among the three sugars in colony forming units (Figure 6 and Table 10).

A stimulatory effect of added sugars on growth was observed when the cells were grown in 5% and 20% oxygen in nitrogen. In NAK medium, CFU at 7 hr was 5.1×10^7 and 2.7×10^7 in 5% and 20% respectively. In the medium with added sugars, CFU at the same hour was 10×10^7 - 87×10^7 per ml in 5 and 20% oxygen in nitrogen. The analysis of variance (Table 10) for CFU showed that the effect of the three atmospheres was highly significant (0.01 level). The effect of atmosphere was highly significant. Time of sampling obviously gave significant differences in growth.

From this study it could be concluded that the added sugar had a slightly stimulatory effect on growth in air and that the sugars had a significant stimulatory effect on growth in 5% and 20% oxygen in nitrogen. The decreased rate of growth in these

atmospheres in the simple NAK medium was significantly relieved when the sugars were added in the medium. This might be due to the presence of intermediates of sugar fermentation during incubation under 5% oxygen. It has been shown that a number of intermediates of carbohydrate fermentation increase growth yield over anaerobic levels by replacing oxygen as the electron acceptor (Hughes and Wimpenny, 1969). However, the increased growth in 20% oxygen in nitrogen when the sugars are added over that in the simple NAK medium cannot be explained.

The effect of the acidic intermediates of carbohydrate metabolism was observed at 7 hr of growth in the cultures with added sugars (Table 9). At this hour, the pH of the culture in simple medium changed very little over the initial value; however, the pH had decreased to 5.9-6.2 in the cultures with added sugars. A similar trend was observed when the cells were grown in 5% and 20% oxygen in nitrogen. Neither the type of sugar nor the atmosphere had a significant effect (Table 10).

Dissolved oxygen concentrations in the media with added sugars during the growth of strain 265-1 in air and in 5% and 20% oxygen in nitrogen are shown in Table 9 and Figure 6. The rate of depletion of DO in the culture medium reflected the growth rate of the cells. The pattern of depletion of DO was quite similar in all three sugar media and the rate of depletion of DO in the

Table 10. Mean values and significance of variance of colony forming units, dissolved oxygen and pH in cultures of *S. aureus* 265-1 growing under 3 atmospheres in media with added sugars.

		Sugar			F ^a	Treatment			F	Sampling Time (hr)			F
		Glucose	Sucrose	Maltose		Air	5% O ₂ in N ₂	20% O ₂ in N ₂		7	11	16	
CFU	(log/ml)	7.15	7.37	7.01	1.68	7.78	6.38	6.93	13.48**	5.68	7.10	8.77	80.50**
	Sugar x Treatment				1.09	Treatment x Sugar			1.09	Time x Sugar			2.93
	Sugar x Time				2.93	Treatment x Time			0.74	Time x Treatment			0.74
DO	(ppm)	3.33	3.1	3.7	0.42	3.4	2.4	3.7	5.27*	5.4	3.3	0.6	0.25
	Sugar x Treatment				2.09	Treatment x Sugar			2.09	Time x Sugar			0.18
	Sugar x Time				0.18	Treatment x Time			4.15**	Time x Treatment			4.15**
pH		6.4	6.5	6.5	1.64	6.4	6.5	6.5	2.72	6.6	6.6	6.2	25.76**
	Sugar x Treatment				2.57	Treatment x Sugar			2.57	Time x Sugar			2.18
	Sugar x Time				2.18	Treatment x Time			4.94**	Time x Treatment			4.94**

a Statistical significance is shown by * for 0.05 level, **, 0.01 level.

culture media was also similar in the three atmospheres. The effect of time was highly significant as would be expected as was the interaction of time with atmosphere (Table 10). The pattern of DO depletion in the culture media substantiated the effect of each sugar on improving growth in 5% and 20% oxygen. In the simple NAK medium, a complete exhaustion of DO was observed at approximately 16 hr (Figure 2) in 5% and 20% oxygen in nitrogen; a complete depletion of DO from the culture media was observed at 7 hr in both when the sugars were added to the medium.

The effect of simple sugars on delaying enterotoxin production by S. aureus in air was observed in this experiment (Table 9).

The production of a detectable amount of enterotoxin A by strain 265-1 was first observed at 7 hr in some of the cultures when sugars were added to the medium, whereas production of enterotoxin was first detected at 3 hr of growth in cultures without added sugars. However, little effect of the sugars on enterotoxin production was observed in the cultures when the cells were grown at 5% and 20% oxygen in nitrogen. Enterotoxin was first detected at 7 hr in the cultures both with and without added sugars.

Morse and Mah (1973) observed in strain 361 in a PHP medium that the repression of enterotoxin B synthesis occurring during oxidative metabolism of glucose was relieved after a shift to anaerobic conditions. It was also observed that aerobic end

products of glucose dissimilation were pyruvate, acetate and lactate whereas the anaerobic end product was mainly lactate. Glucose was catabolized much faster anaerobically than aerobically, presumably to obtain the equivalent amount of energy available aerobically. The presence of a high concentration of lactate in anaerobic glucose breakdown was considered to be evidence that pyruvate was serving as the terminal electron acceptor. From this observation, the investigators postulated that the repressive mechanism is associated with the energy state of the cells. Thus, the effect of 5% oxygen on enterotoxin A synthesis in the NAK media with added sugars observed in this experiment might be attributed to a mechanism similar to that postulated by Morse and Mah.

However, the comparable effect of 20% oxygen in nitrogen on glucose repression of enterotoxin synthesis observed in this study cannot be easily explained. If it was only the availability of oxygen that affected the repression and derepression of enterotoxin synthesis, the effects should have been similar in the culture grown in air and in the culture grown in 20% oxygen in nitrogen.

Implications for further study

A further in-depth study on energetics of staphylococcal metabolism is needed for possible elucidation of the mechanisms of enterotoxin production. The sampling times should be spaced close in time. Because of possible strain differences, the study of more than one strain will be needed in this detail.

The application of the ATP assay method used in this study to food systems might greatly simplify the quantitation of microbial contamination of foods. It has been reported that ATP determination gave a better estimate of bacterial contamination in foods than the plate count method.

It has been postulated that carbon dioxide influences staphylococcal metabolism. Foods may be packaged in materials permeable to carbon dioxide or, in some products, carbon dioxide may be formed by chemical or metabolic reactions. There was an apparent difference in metabolic effect in this study between 20% oxygen in nitrogen and air. For growth in air, the incubation was done in the ambient air; for growth in 20% oxygen in nitrogen, the gas mixture was supplied to the culture media from a pressurized cylinder. However, the dissolved oxygen tension at 0 hr was the same in both. Thus, more study is needed.

SUMMARY

The effect of different oxygen environments on growth and enterotoxin production of S. aureus was investigated. In the first experiment three strains, 265-1, S-6, and 361, producing type A, B, and C enterotoxins, respectively, were used in a 4% NAK medium. Each strain was grown under three different oxygen environments: air, 5% oxygen + 95% nitrogen and 20% oxygen + 80% nitrogen. Colony forming units (CFU), dry weight, dissolved oxygen concentration (DO), pH, energy charge and adenosine phosphates, and enterotoxin were analyzed. The sampling hours were 3, 5, 7, 11 and 16 for the cultures in air and 7, 11, 16, 20 and 24 for the cultures grown in 5% and 20% oxygen in nitrogen. The medium used was 4% NAK supplemented with thiamin and niacin. The effect of sugars on growth and enterotoxin production was investigated in a second experiment using strain 265-1. Glucose, sucrose and maltose were added at a 1% level. CFU, DO, pH, and enterotoxin were analyzed at 3, 5 and 7 hr of growth.

Both as measured by CFU and dry weight, growth was fastest in air for all three strains followed by 20% and 5% oxygen in nitrogen. All the strains reached a CFU of approximately 1×10^{10} cells per ml at the stationary phase of growth under each of the

three atmospheric conditions. However, the duration of the growth phase varied with each atmosphere. The lag phase was more prolonged in 5% and 20% oxygen in nitrogen than it was in air in all of the strains.

A strain difference was observed in the growth curve and in growth response to different atmospheric conditions. The growth curves for strain 265-1 and S-6 were very similar and the growth response to the other two atmospheres was also similar. Strain 361 differed from the other two strains in its growth curve and in growth response to the atmospheres. The lag phase was longer and the exponential phase was steeper in strain 361 in air. The growth of strain 361 was least affected by 20% oxygen in nitrogen.

During growth in air each strain reached a pH of about 7 at 11 hr and 8 at 16 hr. During growth in 5% and 20% oxygen in nitrogen, all the strains reached a pH of 7 between 11 and 16 hr and 8 at 20 and 24 hr.

The depletion of DO in the culture medium closely reflected the growth phase of the cells. During the lag phase, the DO concentration changed very little and was depleted very rapidly during the exponential phase when the cellular oxygen demand was great. The concentration of DO started to increase at the beginning of the stationary phase when the cellular energy need decreased.

There was a definite pattern of change in energy charge during

early phase of the growth cycle of S. aureus with a distinct strain difference. In strain 265-1, the energy charge increased very rapidly and had reached 0.68 at 3 hr of growth. The energy charge in strains S-6 and 361 reached an equivalent peak at 8 hr. In strain S-6 the energy charge decreased to 0.02 during the early hours of growth; in strain 361, it remained at about 0.1 during this period and started to increase rapidly. In both strains, the energy charge started to decrease at the early part of the stationary phase, although the difference was not statistically significant. The time at which enterotoxin production was detectable coincided with a rapid increase in energy charge in strains 265-1 and S-6. The appearance of enterotoxin also coincided with a rapid decrease in dissolved oxygen tension in the culture media.

Strain differences were observed in the time sequence of enterotoxin production in air. Enterotoxin A by strain 265-1 was detected at 3 hr of growth whereas enterotoxin B by strain S-6 and enterotoxin C by strain 361 were detected at 5 and 11 hr, respectively. The amount of enterotoxin produced by each strain varied.

Strain differences of S. aureus in response to environmental conditions were also observed in enterotoxin production. In strains 265-1 and S-6, enterotoxin production was faster and in greater amount in air than it was in either 5% or 20% oxygen in nitrogen. In both of these, enterotoxins A and B were detected at 7 hr of

growth. The response of strain 361 was distinctively different from the other two strains. In this strain, the atmospheres of 5% and 20% oxygen in nitrogen had a stimulatory effect on enterotoxin production. In air, enterotoxin C was not detected until 11 hr of growth; however, in 5% and 20% oxygen in nitrogen, it was detected at 5 hr. The amounts of enterotoxin C produced at 24 hr were similar in the three atmospheres.

Added sugars had a significantly stimulatory effect on growth in 5% and 20% oxygen in nitrogen and a slightly stimulatory effect on growth in air. The decreased growth observed in 5% and 20% oxygen in nitrogen as compared to air when cells were grown in the simple NAK medium was not found when the sugars were added. In NAK-medium, CFU at 7 hr was 71×10^3 and 27×10^3 in 5% and 20% oxygen, respectively. In the medium with added sugars, CFU at the same hour was 10×10^7 - 87×10^7 and 29×10^7 - 80×10^7 per ml in 5% and 20% oxygen in nitrogen, respectively.

The repressive effect of simple sugars on enterotoxin production by S. aureus in air was observed in this experiment. The production of a detectable amount of enterotoxin A by strain 265-1 was observed at 7 hr in some of the cultures when sugars were added to the medium, whereas production of enterotoxin was first detected at 3 hr of growth in cultures without added sugars. However, the repressive effect of the sugars on enterotoxin production was not

observed when the cells were grown in 5% and 20% oxygen in nitrogen. The enterotoxin was detected at 7 hr in both culture media with and without added sugars in all three environments.

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