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MICROBIAL PHYSIOLOGY

Edited by

A. H. ROSE

and

D. W. TEMPEST

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School of Biological Sciences
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D. R. WOODS and D. T. JONES

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Physiological Responses of *Bacteroides* and *Clostridium* Strains to Environmental Stress Factors

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I. Introduction

The two genera *Bacteroides* (non-spore-forming Gram-negative bacilli) and *Clostridium* (endospore-forming Gram-positive bacilli) comprise important groups of obligate anaerobic bacteria. Since bacteria from both genera are

causative agents of disease in humans and animals (Dowell and Lombard, 1981; Hill, 1981), they are among the most intensively studied of the obligate anaerobes.

Within the obligate anaerobic bacteria, members of the genus *Bacteroides* constitute the single most important group of human pathogens and usually occur as mixed infections (Bartlett, 1983; Salyers, 1984). The unique patterns of resistance to antibiotics displayed by these organisms make them difficult to treat (Bawdon *et al.*, 1982). Non-pathogenic strains of *Bacteroides* are the common inhabitants of the alimentary tract of warm-blooded animals and account for a major portion of the bacteria in certain regions of the gut (Macy, 1981). Their importance is emphasized since they inhabit parts of the gastrointestinal tract where there is little or no host digestive capability (e.g. mouth, caecum, colon, rumen) (Gorbach and Levitan, 1970; Hungate, 1966, 1977).

Since the early days of bacteriology, the clostridia have been known to cause diseases in man and domestic animals. The ability of many species to produce potent toxins has given these organisms special significance in the field of public health (Crowther and Baird-Parker, 1983). The clostridia are widely distributed in soils and aquatic sediments (Slepecky and Leadbetter, 1984). They have been reported as inhabitants of the alimentary tracts of both ruminants and non-ruminants, although their role and importance in these environments still remains uncertain. The clostridia, which have diverse fermentative abilities (Nakhmanovich and Shcheblykina, 1959; Barker, 1961; Stadtman, 1973) and produce a variety of organic acids and solvents (Spivey, 1978; Zeikus, 1980; Zeikus, 1980, 1983; Rogers, 1985), have great potential in the fields of biotechnology and industrial microbiology.

In spite of the importance of obligate anaerobes in general, and *Bacteroides* and *Clostridium* strains in particular, studies on these bacteria have tended to lag behind those on aerobes. This disparity is most apparent in the fields of molecular biology and genetics. The major reason for the neglect of the anaerobic bacteria has been the technical difficulties of working with these organisms. However, modern technical developments have overcome many of the difficulties associated with the study of anaerobes. Furthermore, molecular biologists are beginning to appreciate the importance of the genetic diversity of bacteria, and as a result there is a rapidly growing interest in anaerobic bacteria.

Cultures in a state of exponential growth, produced under near optimum conditions, are the stock in trade of the experimental microbiologist (Mandelstam, 1971). They are easily produced and the results obtained with them are fairly reproducible from day to day. It is easy to forget that, in the natural state, periods of exponential growth must be rare and short. Most of the time, bacteria are subjected to environmental stresses and are in a state of partial or total starvation where little or no growth can occur.

A field of research that has resulted in major advances in the molecular biology of aerobes involves their response to environmental stress. Studies on nutrient depletion in bacteria have resulted in an understanding of the stringent response and of the way in which metabolism of the cell is regulated and co-ordinated by enzyme induction and repression and feedback inhibition. Similar studies on the effect of physical stress factors such as radiation and heat have facilitated an understanding of how organisms are able to withstand and repair damage resulting from such environmental stresses. For example, studies on stress induced by UV irradiation and UV-induced phage reactivation have played an important role in the characterization of DNA repair, recombination and mutagenic systems in *Escherichia coli*, *Salmonella typhimurium* and other Gram-negative aerobes (Bernstein, 1981; Walker, 1984). These pioneering studies with UV-irradiation have formed the basis of recent comparative molecular biology studies involving responses to heat, radiation, oxygen, oxidizing agents, methylating agents and ethanol (Lee *et al.*, 1983; Varshansky, 1983; Walker, 1984).

In the endospore-forming Gram-positive bacilli the organization and regulation of metabolism during vegetative growth is similar to non-spore-forming bacteria, but these organisms are able to exhibit the additional response of producing a highly resistant and dormant spore in response to environmental stress. This serves as a means of preserving viability through long periods when the environmental conditions are inimical to growth. Extensive studies of spore formation and germination in *Bacillus subtilis* and a number of other species of *Bacillus* have enhanced our understanding of the regulation of differentiation in prokaryotes (Halvorsen, 1962; Schaeffer *et al.*, 1965; Vinter, 1969; Dawes and Mandelstam, 1970; Young and Mandelstam, 1979).

Molecular biology studies in prokaryotes have been facilitated by the extensive work on the physiological responses of the aerobic bacteria, in particular *E. coli* and *B. subtilis*, to stress factors. Before similar comparative molecular biology studies can be undertaken in anaerobes it is necessary that the basic physiological responses of anaerobes to environmental stress are characterized. In view of the importance of the genera *Bacteroides* and *Clostridium*, and the obvious analogy between *Bacteroides* and the enterobacteria (*E. coli* in particular) and between *Clostridium* and *Bacillus*, we have confined our review to these two anaerobic genera. It is often tempting to regard the responses of *E. coli* and *Bacillus* spp. as typical for non-spore-forming Gram-negative and spore-forming Gram-positive bacteria, respectively. In this review we have emphasized the diversity of bacterial responses to selected stress factors and hope that it will provide a basis for future comparative molecular biology studies.

With the exception of the response of anaerobes to oxygen toxicity,

information regarding the other types of environmentally imposed stress is limited. The environmental stresses that have been chosen for review are those that affect macromolecular synthesis and induce specific gene products in Gram-negative non-spore-forming bacteria, and factors that regulate cellular differentiation and related stationary-phase events in Gram-positive spore formers.

II. Physiological responses of *Bacteroides fragilis* to Stress Factors

The stress factors that have been chosen for emphasis in this section of the review involve agents that, among other activities, are known to damage DNA and result in the induction of specific gene products. Studies on the responses of enterobacteria to DNA-damaging agents have contributed greatly to an understanding of recombination, DNA repair and mutagenesis in Gram-negative aerobes. Although data are still limited at present, evidence suggests that there may be novel and interesting features regarding chromosomal recombination, DNA repair systems and mutagenesis in *Bacteroides*.

Although non-plasmid transfer of antibiotic resistance (conjugative transposition) has been described in *Bacteroides fragilis* (Mays *et al.*, 1981, 1982; Tally *et al.*, 1981; Smith *et al.*, 1982), homologous chromosomal recombination has not been reported for *Bacteroides*. Recombination between a phage genome and the *Bacteroides* chromosome also has not been demonstrated, and despite extensive searches by a member of our laboratory and by Booth *et al.* (1979), lysogeny and transduction have not been shown. Pseudolysogeny appears to be common in *Bacteroides* spp. (Keller and Traub, 1974; Burt and Woods, 1977), but true lysogeny seems to be rare or absent. Silver *et al.* (1975) reported phage-like particles which were observed in thin sections of *B. fragilis* cells associated with larger spherical bodies 300 nm in diameter. Phage heads were always present in association with these bodies, and the intact phages appeared to belong to Bradley's group B. These structures were presumed to be temperate phages although no sensitive strain was found and it was not possible to induce the phage. Lysogenic phages were reported by Prévot *et al.* (1970) and Nacesu *et al.* (1972), but pseudolysogeny was not ruled out in these earlier reports and more recent attempts to isolate prophages have been unsuccessful.

Studies on the mutagenesis of *B. fragilis* and other obligate anaerobes indicate that they are difficult to mutate (Van Tassell and Wilkins, 1978; Droffner and Yamamoto, 1983). Droffner and Yamamoto (1983) investigated the effect of stringent anaerobic conditions on mutagenesis in *S. typhimurium*. Error-prone repair was not expressed in *S. typhimurium* grown under strictly anaerobic conditions. This was due to the lack of expression of *recA* and

recBC functions. They proposed the hypothesis that the anaerobic environment might establish a physiological condition causing the lack of expression or malfunction of the cellular activities necessary for error-prone repair activity in facultative anaerobes.

In an excellent and comprehensive review on the responses of *E. coli* to DNA damage, Walker (1984) identified three regulatory networks that can be induced by exposure to DNA-damaging agents. Ultraviolet radiation and nalidixic acid induce the SOS response which involves the induction of at least 15 different genes and is regulated by the *recA* and *lexA* genes. The heat-shock response is induced by heat and ethanol and at least 13 genes regulated by the *htpR* gene are induced. Methylating agents induce an adaptive response involving at least two genes regulated by the *ada* gene. Studies by Demple and Halbrook (1983) suggest that there may be a fourth regulatory system induced by oxidizing agents and concerned with the repair of oxidative DNA damage. In view of the toxicity of oxygen to anaerobes, the responses of *Bacteriodes* spp. to oxygen and its radicals will be considered first.

A. OXYGEN AND HYDROGEN PEROXIDE

Oxygen is toxic to all living organisms (Haugaard, 1968; Gottlieb, 1971) but bacteria differ in their sensitivity to oxygen and a continuous spectrum of oxygen tolerance, from the most strict anaerobe to the least sensitive hyperaerobe, can be discerned (Morris, 1976). The topic of oxygen toxicity has been reviewed by Fridovich (1975). Morris (1975, 1976), Rolfe *et al.* (1978), Hassan and Fridovich (1979); Privalle and Gregory (1979), Gregory and Dapper (1980), Fee (1982) and Singh and Singh (1982).

Oxygen most commonly exists in the inactivated ground state which is called triplet oxygen ($^3O_2$). Singlet oxygen, which is a higher energy form of oxygen, is very reactive and is one of the forms of oxygen that is toxic to living organisms. The reduction of oxygen to water requires the addition of four electrons. The reduction of $^3O_2$ by a single electron generates the superoxide radical (O_2^-). This radical is highly reactive and can damage DNA by causing single-strand breaks (Moody and Hassan, 1982) either directly or by the secondary production of other radicals (peroxyanion) that attack DNA. Superoxide radicals also cause oxidative destruction of lipids and other biochemical components. The superoxide radical can disproportionate spontaneously to form H_2O_2 and singlet oxygen, and can interact with iron chelates and H_2O_2 in a catalytic cycle to generate the hydroxyl radical (OH^0) which is the most reactive of the various oxygen radicals. Hydroxyl radicals are capable of indiscriminately reacting with and damaging nucleic acids (Cadet and Teoule, 1978; Ito, 1978; Lynch and Fridovich, 1978; Totter, 1981).

They abstract hydrogen atoms from saturated carbon atoms of both purines and pyrimidines at a constant rate close to the limit set by diffusion.

Although anaerobes differ markedly in their sensitivity to oxygen (Fredette *et al.*, 1967; Loesche, 1969; Tally *et al.*, 1975; Walden and Hentges, 1975; Hoshino *et al.*, 1978; Rolfe *et al.*, 1977, 1978), the presence of molecular oxygen inhibits growth of all obligate anaerobes (Loesche, 1969; O'Brien and Morris, 1971; Walden and Hentges, 1975; Hoshino *et al.*, 1978; Rolfe *et al.*, 1978; Wimpenny and Samah, 1978; Samah and Wimpenny, 1982) and prolonged exposure to oxygen invariably leads to cell death. Obligate anaerobes were divided into two groups by Loesche (1969): strict anaerobes, which are unable to grow on plates when there is more than 0.5% oxygen in the atmosphere, and moderate anaerobes, which can grow on plates in the presence of 2–5% oxygen. Certain factors may, however, affect the classification of anaerobes into the different groups: several strict anaerobes may become more aerotolerant after two or three subcultures (Willis, 1969), and cells in the exponential growth phase in batch culture are usually more oxygen-sensitive than cells in the stationary phase (Morris, 1976; Jones *et al.*, 1980a). Loesche (1969) reported that *B. fragilis* can withstand exposure to air for periods of at least 60 minutes without loss of viability, and Onderdonk *et al.* (1976) reported that the effect of oxygen on *B. fragilis* cells growing in chemostat culture is only bacteriostatic in nature. Jones and Woods (1981) isolated a *B. fragilis* Bf-2 strain which was maintained in various aerobic holding solutions for 1 to 6 hours without loss in viability.

The survival of anaerobes in air may be assisted by oxygen free radical scavenging enzymes. McCord and Fridovich (1969) were the first to report the existence in bacteria of superoxide dismutase (SOD) enzymes. Superoxide dismutase very efficiently catalyses the safe dismutation of superoxide radicals to yield hydrogen peroxide and triplet oxygen (Morris, 1976). This enzyme also can effectively quench singlet oxygen (Fridovich, 1974), and in *E. coli* cells a definite correlation seems to exist between oxygen sensitivity and a lack of SOD production in mutants of this organism (Hassan and Fridovich, 1979).

In 1971, McCord *et al.* proposed the superoxide dismutase theory of obligate anaerobiosis in which they stated that the lack of this important enzyme accounted for the aero-intolerance of anaerobes. The production of this enzyme is, however, not restricted to aerobic and aerotolerant bacteria and a few years after McCord *et al.* (1971) proposed their theory, SOD was discovered in several aero-intolerant and strictly anaerobic bacteria. These bacteria often contain at least two electrophoretically distinct SOD enzymes (Hewitt and Morris, 1975; Ashley and Shoesmith, 1977; Gregory *et al.*, 1978). The aero-intolerant bacteria in which the presence of SOD has been demonstrated include *Chlorobium*, *Desulfovibrio*, *Clostridium* (Morris, 1976) and several *Bacteroides* species (Carlsson *et al.*, 1977; Gregory *et al.*, 1977a;

Tally *et al.*, 1977; Rolfe *et al.*, 1978). In *Bacteroides* spp. (Carlsson *et al.*, 1977; Tally *et al.*, 1977), *Clostridium* spp. (Ashley and Shoesmith, 1977) and *Selenomonas ruminantium* (Wimpenny and Samah, 1978; Samah and Wimpenny, 1982) the levels of SOD present in the cells correlate well with the oxygen tolerance of these organisms. The SOD level in certain *Bacteroides* species is similar to, or even greater than, that reported for *E. coli* (Gregory *et al.*, 1978).

The presence of SOD in strict anaerobes has been explained in different ways. The most plausible one is that the presence of SOD enables them to survive transient exposures to oxygen, albeit with growth inhibition (Tally *et al.*, 1977; Halliwell, 1982). Lumsden and Hall (1975) considered that, in anaerobes, SOD provides protection against superoxide radicals formed by cosmic radiation, while Tally *et al.* (1977) proposed that SOD is used as a virulence factor by pathogenic anaerobes.

There are four different types of SOD enzymes known. Copper- and zinc-containing SOD enzymes (Cu/Zn SOD) are characteristic of the cytosol of eukaryotic cells, although similar enzymes have been found in the prokaryotes *Photobacterium leioyhathi* and *Micrococcus denitrificans* (Bannister and Bannister, 1985). Manganese SOD enzymes (MnSOD) are common to both prokaryotes (Vance *et al.*, 1972; Weisiger and Fridovich, 1973) and eukaryotes (Bannister and Bannister, 1981), but SOD enzymes containing iron (FeSOD) have so far only been isolated from prokaryotes (Bannister and Bannister, 1981). In *E. coli*, MnSOD is found in the cell matrix (Keele *et al.*, 1970; Gregory *et al.*, 1973) and presumably protects the organism against endogenously produced superoxide radicals (Rolfe *et al.*, 1978), while FeSOD is found in the periplasmic space (Gregory *et al.*, 1973; Yost and Fridovich, 1973) and may protect against exogenously generated radicals. This latter type of protection may be important for anaerobes that reduce little or no oxygen internally, and will also be advantageous in the establishment of infections in well oxygenated tissues (Rolfe *et al.*, 1978).

The level of SOD in *E. coli* can be induced by oxygen to 25 times the anaerobic level (Gregory and Fridovich, 1973a), while the maximum level of SOD in aerobic *Streptococcus faecalis* is 16-fold higher than that present in the cells during anaerobic growth (Gregory and Fridovich, 1973a). An increase in intracellular SOD correlates well with a gain in resistance to hyperbaric oxygen in *E. coli*, *S. faecalis* and *Saccharomyces cerevisiae* (Fridovich, 1972, 1974, 1975; Gregory and Fridovich, 1973a,b, 1974; Hassan and Fridovich, 1977). *Bacteroides* species contain a low level of SOD in cells grown under anaerobic conditions: when these cells are exposed to 2% oxygen, growth is inhibited and the SOD content increases to five times the anaerobic level (Gregory *et al.*, 1977b).

One of the by-products of oxygen reduction by NADH oxidase is the

superoxide radical (Wimpenny and Samah, 1978). In the anaerobe *Selenomonas ruminantium* a SOD enzyme is induced at the same time as the NADH oxidase and complements the NADH oxidase action in protecting these organisms against oxygen toxicity (Wimpenny and Samah, 1978; Samah and Wimpenny, 1982). Wimpenny and Samah (1978) reported that the inducible SOD responded to higher oxygen concentrations than the NADH oxidase and that SOD levels were highest in cells whose growth was completely inhibited by oxygen. In 1982, however, they modified this statement and reported that the activity of SOD declines at high oxygen concentrations. The induction of NADH oxidase reaches a maximum at a higher aeration level than the aeration level necessary to evoke maximum SOD production. Both NADH oxidase and SOD activities decrease at oxygen concentrations high enough to inhibit growth in *Selenomonas ruminantium* (Samah and Wimpenny, 1982).

Hydrogen peroxide is one of the most stable oxygen radicals and is produced in most aerobically grown cells (Lemberg and Legge, 1949) through the two-electron reduction of oxygen which is generally mediated by reduced flavoproteins (Malström, 1982). Furthermore, H_2O_2 is almost ubiquitous in autoclaved culture media exposed to air. The heating of glucose and phosphate together in culture media produces intermediates that generate H_2O_2 on aeration of the media (Carlsson *et al.*, 1978). The oxidation of the thiol group of the cysteine present in anaerobic media also produces H_2O_2 when these media are exposed to air (Carlsson *et al.*, 1969).

An increased number of single-strand DNA breaks and enhanced lethality were observed in *E. coli* (Pollard and Weller, 1967; Ananthaswamy and Eisenstarck, 1977; Hartman and Eisenstarck, 1978; Carlsson and Carpenter, 1980), *S. typhimurium* (Yoakum and Eisenstarck, 1972; Carlsson and Carpenter, 1980), and T7 phage (Ananthaswamy and Eisenstarck, 1976), after treatment with H_2O_2. Hydrogen peroxide affects isolated DNA by altering it such that all four bases are liberated (Uchida *et al.*, 1965; Yamafugi and Uchida, 1966; Freese *et al.*, 1967; Rhaese and Freese, 1968; Massie *et al.*, 1972). All studies on isolated DNA, however, were carried out using relatively high concentrations of H_2O_2 (0.05 to 0.1 M) and long periods of incubation in the presence of ferric chloride. In heat-treated bacteria, H_2O_2 appears to interfere with the ability of the cells to recover after stress (Martin *et al.*, 1976; Brewer *et al.*, 1977; Flowers *et al.*, 1977; Rayman *et al.*, 1978). It also displays a synergistic effect with near-UV irradiation and Hartman and Eisenstarck (1978) proposed that H_2O_2 decreases *recA*$^+$-dependent repair of UV-induced DNA damage. The latter synergism was thought to be specific for near-UV irradiation (Hartman and Eisenstarck, 1978), but Bayliss and Waites (1979) found that the simultaneous treatment of dormant spores of *Bacillus subtilis* with far-UV radiation and H_2O_2 resulted in a 2000-fold greater kill than that

produced by irradiation alone or followed by a treatment with H_2O_2. This synergism was not due to the hydroxyl radicals formed during the decomposition of H_2O_2 by UV irradiation, since hydroxyl radical quenchers failed to protect the spores.

It was thought that the possession of the H_2O_2 scavenging enzymes, catalase and/or peroxidase, is essential for survival of living organisms in air (Saunders, 1973; Stadtman, 1980) and that the lack of these enzymes in anaerobes accounts for their oxygen sensitivity. Holdeman and Moore (1972) showed, however, that a large number of obligate anaerobes produce these enzymes and Hansen and Stewart (1978) recently recommended a catalase test as a rapid screening procedure to identify members of the "*B. fragilis*" group of anaerobic Gram-negative bacilli.

Bacteroides distasonis is the anaerobe that produces the most catalase. The catalase concentrations reported for this organism are comparable with those observed in aerobic *E. coli* (Gregory and Fridovich, 1973b) and aerotolerant *Streptococcus faecalis* organisms (Gregory and Fridovich, 1973a). The *B. distasonis* catalase enzyme has a molecular weight of 250,000 and its uninduced production reaches peak values in the late logarithmic growth phase (Gregory *et al.*, 1977b).

Gregory and Fridovich (1973b) claimed that catalase is not induced by oxygen in *E. coli* cells, but Hassan and Fridovich (1977) reported a 2.4-fold increase in catalase and a 12.7-fold increase in peroxidase levels on aeration of *E. coli* K12 cells. The induction of catalase by oxygen in anaerobes is markedly influenced by the growth medium.

Catalase is a non-dialysable, cyanide- and azide-sensitive heat-labile protein (Gregory *et al.*, 1977b) which, with a few exceptions (Johnston and Delwiche, 1965), contains heme as a biologically active-centre component (Bomberg and Luse, 1963; Schonbaum and Chance, 1976). *Bacteroides fragilis* cannot synthesize its own heme and must therefore transport into the cell preformed hemin for synthesis of heme proteins like catalases and cytochromes (Sperry *et al.*, 1977). Several other *Bacteroides* species also require preformed hemin for catalase production, and catalase levels of *B. distasonis* were found to vary with the amount of hemin supplied in the medium (Gregory *et al.*, 1977b). Ten times as much hemin is required for catalase production than for optimum growth (Wilkins *et al.*, 1978). Since it is heme availability, and not total heme concentration, that is the important factor in determining catalase production, higher concentrations of catalase can be induced in some media if hemin is added after autoclaving. The effect of hemin on catalase production is very specific and cannot be duplicated by ferrous sulphate or ferrous ammonium citrate (Gregory *et al.*, 1977b). Vitamin K apparently acts synergistically with hemin in elevating the catalase-specific activity (Gregory *et al.*, 1977b). The requirement for exogenously

supplied hemin was not recognized until recently, and is one of the reasons why catalase production in anaerobes remained undetected for such a long time.

Gregory *et al.* (1977a) showed that catalase production and induction in a number of *Bacteroides* species is suppressed by the presence of glucose and other carbohydrates in the medium. When cells, grown in the absence of carbohydrates, are transferred to media containing glucose, the residual catalase activity already in the cells is not destroyed, but is diluted out as the cells multiply without producing more enzyme. In the absence of carbohydrates, *B. fragilis* produces relatively large amounts of catalase (25–50 units (mg protein)$^{-1}$; Gregory *et al.*, 1977a). Anaerobes are routinely cultured in media that contain carbohydrates as energy sources and this may be another reason why catalase production was not previously detected in these organisms.

Several investigators have stated that catalase induction does not render cells more tolerant towards oxygen (Gregory and Fridovich, 1973a,b; Fridovich, 1974; Rolfe *et al.*, 1978) or less sensitive to H_2O_2 (Adler, 1963; Ananthaswamy and Eisenstark, 1977). Although catalase significantly lowers the concentration of H_2O_2 present in cells during aerobic metabolism (Schonbaum and Chance, 1976), its activity *in vivo* is affected by several environmental conditions. The endogenous catalase in thermally stressed *Staphylcoccus aureus* cells is inactivated by the combined action of heat and NaCl in selective *Staphylococcus* media; exogenously added catalase was found to cause as much as a 15,000-fold increase in the enumeration of these thermally stressed *Staphylococcus aureus* cells (Martin *et al.*, 1976). Catalase also is inactivated both by high concentrations of H_2O_2 and by cysteine, the latter being a standard component of anaerobic media (Boeri and Bonnichsen, 1952; Alexander, 1957). Peroxidase enzymes, which are effective scavengers of H_2O_2 and are capable of using cysteine as a substrate in the reaction (Olsen and Davis, 1976), are therefore much better suited to protect anaerobes against the bacteriocidal effect of aerated cysteine-containing media (Carlsson *et al.*, 1969).

B. EFFECT OF OXYGEN AND HYDROGEN PEROXIDE ON MACROMOLECULAR SYNTHESIS

Although numerous reports have shown that oxygen inhibits the growth of obligate anaerobes, this does not necessarily imply that macromolecular synthesis is immediately blocked. There are examples of stationary phase Gram-negative aerobic bacteria which continue to synthesize proteins and nucleic acids for long periods (weeks) after growth has ceased (Robb *et al.*, 1980). Furthermore, aeration of these cells affects the rate of synthesis of nucleic acids and proteins but does not totally inhibit their production.