THE CARBON DIOXIDE REQUIREMENTS OF ANAEROBIC BACTERIA

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PLATE XXXIX

FOR ALMOST a century, experimental evidence concerning the importance of carbon dioxide for bacterial growth has been accumulating (Winogradsky, 1890; Rockwell, 1923; Rockwell and Highberger, 1927; Valley and Rettger, 1927; Valley, 1928; Gladstone, Fildes and Richardson, 1935; Wimpenny, 1969). The attention of early workers was directed primarily at aerobes such as *Neisseria gonorrhoeae* (Chapin, 1918), *N. meningitidis* (Cohen and Fleming, 1918), *Brucella abortus* (Smith, 1924; Wilson, 1931) and *Mycobacterium tuberculosis* (Wherry and Ervin, 1918; Davies, 1940). With the recent resurgence of interest in anaerobic bacteriology, the essential or stimulatory effect of CO₂ on the growth of obligate anaerobes has become widely recognised. Current anaerobic culture techniques commonly employ 5–10% CO₂ in the incubation atmosphere to achieve optimal growth (Watt, 1973; Stalons, Thornsberry and Dowell, 1974; Willis, 1977). Nevertheless, there is a lack of precise information relating to the quantitative CO₂ requirements of many clinically important anaerobic bacteria. Preliminary experiments in this laboratory revealed a wide variation in the CO₂ requirements of different species of anaerobic bacteria; the observation that relatively low concentrations of CO₂ were adequate for satisfactory growth of certain anaerobes was of particular interest. The present study reports the effect of different concentrations of CO₂ on the growth of a range of anaerobic bacteria isolated from clinical sources.

MATERIALS AND METHODS

Micro-organisms. The following organisms were examined: 57 strains of *Bacteroides* spp. isolated from faeces, comprising *B. fragilis* (20 strains), *B. vulgatus* (5 strains), *B. distasonis* (10 strains) and *B. thetaiotaomicron* (22 strains); 30 strains of *B. melaninogenicus* freshly isolated from the upper respiratory tract; 11 strains of *Fusobacterium* spp., comprising *F. varium* (6 strains), *F. necrophorum* (3 strains) and one strain each of *F. necrogenes* and *F. gonidiaformans*; 23 strains of *Clostridium perfringens*; 19 strains of *C. difficile*; 4 strains of *C. innocuum*; 2 strains of *C. bifermentans*; one strain each of *C. chauvoei*, *C. histolyticum*, *C. sporogenes* and *C. septicum*; 14 strains of *Bifidobacterium* spp.; 12 strains of anaerobic cocci, comprising *Peptococcus asaccharolyticus* (7 strains), *P. magnus* (1 strain), *P. prevoti* (1 strain), *Peptostreptococcus anaerobius* (2 strains) and *Pst. micros* (1 strain). Identification of isolates was by the methods of Holdeman, Cato and Moore (1977).

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Culture medium. Blood-agar Plates No. 2 (LAB-M Ltd, Salford, Lancs) containing 7% horse blood were used.

Inoculation of medium. Suspensions of each organism were prepared in sterile saline. The approximate concentrations of cells were as follows: *Bacteroides* spp. and *Fusobacterium* spp., 10^5 /ml; *B. melaninogenicus*, 10^6 /ml; *Clostridium* spp., 10^7 /ml; *Bifidobacterium* spp. and anaerobic cocci, 10^8 /ml. A multipoint inoculator was used to seed blood-agar plates with 0.001 ml of each suspension. A 9-cm petri dish conveniently accommodated 20 cultures.

Atmospheric conditions. Standard Baird and Tatlock 3-litre jars, equipped with Schraeder valves, were fitted with 4 g of freshly activated palladium catalyst before each experiment. Pure CO₂ and H₂ were obtained from separate cylinders supplied by the British Oxygen Company. Each jar was evacuated to an internal pressure of 40 mm Hg, i.e., 720 mm Hg below atmospheric pressure; residual CO₂ in the atmosphere was considered to be negligible. The appropriate volume of CO₂ was introduced from a balloon. Accurate measurement of small volumes of CO₂ was achieved by drawing the gas into a graduated syringe and injecting it into the side arm of the anaerobic jar. Volumes of CO₂ in excess of 10% were admitted via the Schraeder valve and monitored manometrically, care being taken to flush through all connecting tubing with CO₂. Internal and external pressures were then equilibrated by the introduction of pure H₂. The following concentrations of CO₂ by volume were chosen: 0.25%, 0.5%, 0.75%, 1%, 2%, 5%, 10%, 20%, 40%, 60% and 80%. For comparative purposes, cultures were incubated in parallel in the anaerobic atmosphere currently in use in the routine clinical laboratory, which was 7% CO₂ in H₂.

Three additional atmospheres were provided to give information on the effect of elimination of CO₂ on bacterial growth: one jar was evacuated and then filled with pure H₂; an open petri dish containing 2 ml of 1N NaOH solution was placed inside a similar jar filled with H₂. Any difference in growth between these two atmospheres was attributed to the absorption by NaOH of residual CO₂ not eliminated by the evacuation-replacement procedure and of CO₂ evolved during bacterial metabolism. Because preliminary experiments demonstrated enhancement of growth in the presence of 0.25% CO₂, a third jar was included containing this concentration of CO₂ plus 2 ml of 1N NaOH and it was incubated in parallel with a jar containing 0.25% CO₂ but devoid of NaOH, to verify the efficacy of NaOH in removing exogenously supplied and endogenously produced CO₂.

All cultures were incubated at 37°C for 48 h with the exception of those for *B. melaninogenicus* which remained undisturbed for 72 h.

Assessment of growth. Bacterial growth in each atmosphere was compared in terms of numbers of colonies and individual colony size; in addition, variations in haemolysis, pigmentation and fluorescence were noted when applicable.

RESULTS

The results obtained are summarised in fig. 1.

Bacteroides spp. (excluding *B. melaninogenicus*). No strain grew in atmospheres from which CO₂ had been totally removed by the inclusion of NaOH, but scanty growth of a few strains was evident in the gaseous environment in which the CO₂ concentration was considered negligible by the evacuation-replacement procedure. Supplementation of the atmosphere with 0.25% CO₂ resulted in good growth of all strains; this was not further improved by incubation in concentrations of CO₂ up to 40%. Increasing the CO₂ concentration above 40% resulted in considerable inhibition of growth, and in general only strains of *B. fragilis* were consistently capable of growth, albeit impaired, in 80% CO₂.

B. melaninogenicus. No strain grew in the presence of NaOH or in an atmosphere lacking exogenous CO₂. Scanty growth of some strains was just discernible after incubation in 0.25% CO₂ for 72 h; increasing the CO₂ content

FIG. 1.—Growth of anaerobic bacteria in different concentrations of CO₂. Figures in parentheses indicate number of strains tested. □ = No growth; ▨ = scanty growth; ▩ = moderate growth; ■ = good growth. Eight strains of *B. melaninogenicus* failed to grow in 0.25% CO₂, three of which failed to grow in 5% CO₂.

to 10% produced consistently good growth of all strains. Incubation of cultures in concentrations of CO₂ up to 40% resulted in further increase in the size of individual colonies and considerable enhancement of haemolysis (figs. 2-5). Atmospheres containing 60% CO₂ were inhibitory to many strains of *B. melaninogenicus*, colonies being smaller, more deeply pigmented and with reduced zones of haemolysis. The characteristic brick-red fluorescence of this species under a Wood's lamp was apparently unaffected by the variations in atmospheric CO₂ content.

Fusobacterium spp. Absorption of CO₂ by NaOH inhibited growth of all strains of fusobacteria. Only *F. varium* and *F. necrogenes* were capable of growth in an atmosphere of H₂ unsupplemented with CO₂. Increased concentrations of CO₂ did not appear to stimulate these species and growth was unimpaired by 80% CO₂. In contrast, *F. necrophorum* required at least 1% CO₂ for growth, and the tolerance of this species to CO₂ declined in atmospheres containing > 20% CO₂. Good growth of the single strain of *F. gonidiaformans* occurred in the range 0.25%-20% CO₂.

C. perfringens. All strains examined grew equally well in the presence or absence of CO₂. Enhancement of growth was not produced by increasing concentrations of CO₂, but haemolysis due to α -toxin was progressively intensified.

C. difficile and *C. innocuum*. If CO₂ was absorbed from the atmosphere, growth of *C. difficile* was moderate, but the colonies were small and irregular. Recovery of *C. innocuum* was not achieved in the presence of NaOH. Both species exhibited moderate growth in an atmosphere of H₂ devoid of exogenously supplied CO₂, but were enhanced by the addition of 0.25% CO₂; further supplementation did not improve growth. Indeed, *C. difficile* grew poorly in exogenous CO₂ concentrations in excess of 40%. Moderate growth of *C. innocuum* persisted in the presence of 80% CO₂.

C. bifermentans, *C. chauvoei* and *C. histolyticum*. Absorption of CO₂ resulted in considerable impairment of growth. All species grew well in the presence of H₂ without additional CO₂ and growth was not improved by increasing the concentrations of CO₂. Atmospheres containing > 40% CO₂ caused marked growth inhibition of all species.

C. sporogenes and *C. septicum*. Growth of these two species was unaffected by absorption of atmospheric CO₂ and by high CO₂ concentrations.

Bifidobacterium spp. With one exception, all 14 strains of bifidobacteria grew well in an atmosphere of H₂ without supplementary CO₂; none grew in the presence of NaOH. Slightly improved growth followed the addition of 0.5% CO₂ to the atmosphere of the jars. Luxuriant growth occurred in all higher concentrations of CO₂ and was not diminished in 80% CO₂.

Anaerobic cocci. Absorption of atmospheric CO₂ permitted only scanty growth of a few strains, but all grew well in H₂ devoid of exogenous CO₂ and were unaffected by further increases in CO₂.

DISCUSSION

One of the requirements for the isolation and identification of anaerobic

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FIG. 2.—Blood-agar plate inoculated with 20 strains of *B. melaninogenicus*, after 72 hours' incubation in 0.25% $\text{CO}_2 + \text{H}_2$. Note absence of some strains, scanty growth of others and weak haemolysis.

FIG. 3.—Identical strains of *B. melaninogenicus* after 72 hours' incubation in 5% $\text{CO}_2 + \text{H}_2$. Note improved growth of some strains, absence of others and enhanced haemolysis.

FIG. 4.—Identical strains of *B. melaninogenicus* after 72 hours' incubation in 7% $\text{CO}_2 + \text{H}_2$. Note good growth of all strains, improved haemolysis and pigmentation of some strains.

FIG. 5.—Identical strains of *B. melaninogenicus* after 72 hours' incubation in 10–40% $\text{CO}_2 + \text{H}_2$. Note optimal growth of all strains and large zones of haemolysis.

bacteria in the routine clinical laboratory is the presence of an optimal atmosphere. This is of particular importance for fastidious species such as *B. melaninogenicus*, which may be isolated from most ecological sites in the human body after prolonged incubation of clinical specimens. Watt (1973) reported optimal isolation of gram-negative anaerobes and of *C. tetani* and *C. oedematiens* types A and B, after incubation in 10% CO₂, and it has since become generally accepted that 5–10% CO₂ is a suitable concentration for satisfactory isolation of the majority of anaerobic bacteria.

In this study, the CO₂ requirements of an extended range of clinically significant anaerobic bacteria have been critically examined. It is appreciated that in any such examination, the results will be influenced by several factors, for example, the age of the cultures, the type of medium, the inoculum size, the duration of incubation, and pH changes in the medium. No attempt was made to investigate the latter variable in this work. Agar containing horse blood 7% (v/v) was used throughout to facilitate comparisons with routine isolation techniques.

The results confirm the wide range in the CO₂ requirements of anaerobic bacteria. A spectrum of CO₂ requirements was demonstrated, ranging from *C. perfringens*, *C. sporogenes* and *C. septicum*, the growth of which was apparently unaffected by the concentration of CO₂ in the atmosphere, to *B. melaninogenicus* which required 10–40% CO₂ for optimal growth. In accordance with preliminary observations, supplementation of the atmosphere with 0.25% CO₂, the lowest concentration achievable with accuracy, was found to satisfy the CO₂ requirements of most of the anaerobes studied, *B. melaninogenicus* being the notable exception. This growth-stimulating effect was abolished by including NaOH in identical jars supplied with 0.25% CO₂ and incubated in parallel. It is interesting that many other species of anaerobic bacteria were, however, capable of some growth when the jar was evacuated to an internal pressure of 40 mm Hg and the vacuum abolished by replacement with pure H₂. This suggests that the residual atmospheric CO₂ was utilised by these species.

Anaerobic bacteria exhibited an analogous diversity of response when exposed to high concentrations of CO₂. Consistent inhibition of growth was observed when *B. thetaiotaomicron*, *B. vulgatus*, *B. distasonis*, *F. necrophorum*, *C. difficile*, *C. bifermentans*, *C. chauvoei* and *C. histolyticum* were subjected to exogenous CO₂ concentrations exceeding 40%. The quality of growth of all strains of *B. melaninogenicus* was optimal in the presence of 40% CO₂; further increase in the CO₂ content resulted in a marked decline in colony size and numbers. In contrast, the strains of *F. varium*, *F. necrogenes*, bifidobacteria and anaerobic cocci continued to grow luxuriantly in all concentrations of CO₂ above 40%. As observed by Watt (1973), growth of *C. perfringens* remained unimpaired in 80% CO₂.

CO₂ plays an intricate role in bacterial metabolism (Walker, 1932; Ajl and Werkman, 1948; Charles and Roberts, 1968; Wimpenny, 1969). Dehority (1971) demonstrated an absolute requirement for CO₂ by those anaerobic rumen bacteria that produce succinic acid as one of their primary end-pro-

ducts. Many other facultative and obligate anaerobes depend on CO₂ for the initiation of growth. From the foregoing results, *B. melaninogenicus* appears to occupy a unique position among this selection of anaerobic bacteria; an exogenous supply of at least 10% CO₂ is a prerequisite for its successful isolation if incubation is continued for only 72 h. It is well recognised that *B. melaninogenicus* is difficult to maintain in pure culture, and CO₂ may be an important factor in the apparent synergism between this species and other anaerobes with which it is commonly associated. The observation that higher concentrations of CO₂ (up to 40%) may further enhance the colonial appearance of *B. melaninogenicus* could be utilised by the microbiologist with a particular interest in the isolation of these fastidious, slowly growing anaerobes. If the CO₂ concentration does not exceed 40%, the isolation of other anaerobic bacteria from clinical specimens would not be appreciably compromised.

It was not intended that this should be an exhaustive study of the CO₂ requirements of all anaerobic bacteria, but rather an examination of the influence of varying atmospheric concentrations of CO₂ on the growth of a selection of anaerobes of clinical interest. The effect of CO₂ on spore germination was not considered. In view of the considerable range of CO₂ requirements demonstrated, the current practice of adding 10% CO₂ to the atmosphere of anaerobic jars is to be encouraged; it stimulates optimal growth of all genera of anaerobic bacteria, thus facilitating routine isolation and identification.

SUMMARY

Some clinically significant anaerobic bacteria were incubated in pure culture in anaerobic jars containing a range of atmospheric concentrations of CO₂. Growth of *Clostridium perfringens*, *C. sporogenes* and *C. septicum* was independent of the amount of CO₂. Small supplements of CO₂ (0.25%) allowed good growth of the majority of anaerobes studied—an observation contrary to established teaching. The exceptions were *Fusobacterium necrophorum* which showed an absolute requirement for CO₂ of at least 1% and *B. melaninogenicus* which needed an atmospheric content of 10–40% CO₂ for optimal growth. The inclusion of CO₂ in the anaerobic jar at a final concentration of 10% is to be recommended for all routine isolation procedures.

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