THE INFLUENCE OF CARBON DIOXIDE ON THE GROWTH OF OBLIGATE AND FACULTATIVE ANAEROBES ON SOLID MEDIA

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THE addition of carbon dioxide (CO₂) to the gas mixture in anaerobic jars has been recommended by several workers (e.g., Willis, 1964; Hobbs, Williams and Willis, 1971), and is a routine step in anaerobic procedure in this laboratory (Collee *et al.*, 1972). However, the basis for this is empirical. Although the stimulatory effect of CO₂ on spore germination is well documented (Wynne and Foster, 1948; Roberts and Hobbs, 1968; Holland, Barker and Wolf, 1970), some workers have noted that it may have an inhibitory effect on the growth of *Clostridium welchii* from spores (Futter and Richardson, 1971). The present paper describes quantitative studies designed to evaluate the importance of added CO₂ in anaerobic jar procedure, with special reference to the recovery of essentially vegetative inocula on solid media.

MATERIALS AND METHODS

Strains. The following test strains were subcultured in cooked-meat broth (CMB): *Clostridium welchii* (*Cl. perfringens*) type A, β -haemolytic, classical (heat-sensitive) strains, laboratory nos. L2A and C1, and NCTC8588; *Cl. welchii* type A, non-haemolytic heat-resistant strains no. NCTC8359, NCTC8238 and NCTC8239; *Cl. histolyticum* no. NCTC503; *Cl. bifermentans* lab. no. B4; *Cl. septicum* lab. no. 101; *Cl. tetani* type VI, no. NCTC9569; *Cl. tetani* no. NCTC5405; *Cl. oedematiens* (*Cl. novyi*) type A, Glaxo no. 5277/64; *Cl. oedematiens* type B, Glaxo no. 5281/64; *Cl. oedematiens* type D, no. NCTC8145; *Bacteroides fragilis* no. NCTC9343; *B. necrophorus* nos. NCTC7155 and NCTC10575; *B. melaninogenicus* nos. NCTC9336 and NCTC9337; and *Bacteroides*-like organisms isolated in Edinburgh from faeces or wound exudates and labelled Gram-negative anaerobic bacilli (GNAB) nos. 1-20. Five strains of *Streptococcus pyogenes* (Lancefield group A) were isolated from clinical material and subcultured in nutrient broth (NB).

Media. Cooked-meat broth was prepared as described by Cruickshank (1968) but the infusion broth component was made from Nutrient Broth No. 2 (Oxoid) which was also used alone for the subculture of some of the test strains. "Pre-steamed nutrient broth" was used as a diluent in quantitative studies; it was heated by steaming at 100°C for 30 min. to remove dissolved air, then cooled rapidly to 37°C.

Blood-agar plates were prepared with Oxoid Blood Agar Base No. 2 enriched with equine blood (10 per cent.). Watt's modification of Moore's medium (cysteine-dithiothreitol equine blood agar, CDEBA) was used for the surface growth of *Cl. oedematiens* strains (Watt, 1972). All plates contained 1.2 per cent. agar unless specified otherwise in the text.

Blood. Defibrinated equine blood was supplied by Wellcome Laboratories.

Anaerobic jar procedure. In the present study, Baird and Tatlock (BTL) anaerobic jars were used. Each jar was equipped with three room-temperature catalyst sachets. The detailed anaerobic jar procedure was basically that described by Collee *et al.* (1972); in addition, jars were evacuated again after being filled with a mixture of 90 per cent. hydrogen

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(H₂) and 10 per cent. carbon dioxide (CO₂) and then refilled with this gas mixture before incubation. All cultures were incubated at 37°C.

Gases. Cylinders of CO₂ were supplied by Distillers Company Ltd. Cylinders of H₂ and a special gas mixture of H₂ 90 per cent. with CO₂ 10 per cent. were supplied by British Oxygen Co. Ltd.

Counting methods. Viable counts performed by a spread-plate method (Watt) were calculated from the mean result obtained from ten plates seeded from a predetermined dilution that gave 100–700 discrete colonies per plate.

Antiserum. An experimental antiserum no. Ex.3567, supplied by Wellcome Research Laboratories, was used to control the growth of the *Cl. septicum* strain (Watt).

RESULTS

Enhancement of recovery of laboratory strains with carbon dioxide

Preliminary studies indicated that enhancement of growth of certain anaerobes, notably Gram-negative anaerobes, occurred if 10 per cent. carbon dioxide was added to the hydrogen gas used in anaerobic jars purchased from Baird and Tatlock Ltd ("BTL jars"). Both the size and number of colonies were increased, but there was no further enhancement with concentrations of CO₂ above 10 per cent. and the enhancement was less marked with concentrations of CO₂ below 10 per cent. A series of statistically designed experiments was therefore developed to compare the recovery of clinically important anaerobes in BTL jars with and without the addition of 10 per cent. CO₂ in the gas mixture.

Appropriate dilutions (see *Materials and methods*) of the test broth cultures were made in pre-steamed nutrient broth and were used to seed replicate plates of freshly poured 10 per cent. equine blood agar (EBA). The seeded plates were randomly distributed between two or more BTL anaerobic jars and the position of each plate within a given jar was also randomised. Half of the jars were processed with added CO₂ and half with H₂ only; the order of processing of the jars was randomised. All the jars in a given experiment were incubated for identical periods of time.

Of the clostridia tested, the recovery of *Cl. tetani* was significantly improved by the addition of 10 per cent. CO₂ (table I), as was the size of colony observed with this non-motile strain. Similar results were obtained with type-A and type-B strains of *Cl. oedematiens* grown on CDEBA medium with 4 per cent. agar and the zones of haemolysis were increased. None of the other clostridia tested showed significant enhancement of recovery with added CO₂.

The results of experiments with *Bacteroides* strains (table II) show that addition of CO₂ to the incubation environment markedly increased the number and size of colonies grown on EBA medium. Although easily visible colonies were obtained after overnight incubation in H₂+CO₂, at best only pin-point colonies were observed on plates incubated in H₂, and incubation for a further 48 hr was necessary before countable colonies were obtained on these plates.

Twenty strains of Gram-negative strictly anaerobic bacilli ("Bacteroides-like organisms"), isolated from wounds and from human faeces and not further characterised, were then tested as described above. All 20 strains showed good growth with development of countable colonies after incubation for 18 hr in

H₂+CO₂. When incubated in H₂ without CO₂, two strains showed no growth even after prolonged incubation and the remaining 18 strains required incubation for up to 96 hr for the development of countable colonies. Of these 18

TABLE I

Recovery of a strain of Clostridium tetani from a dilution of a CMB culture grown anaerobically on EBA medium in BTL jars with and without carbon dioxide*

Test culture	Jar number	Gas used	Mean colony count±S.E.†	t-value‡
18-hr CMB culture of <i>Cl. tetani</i> type VI, no. NCTC9569	C7	90 per cent. H ₂ +10 per cent. CO ₂	1014.0±27.8	3.06 (P<0.01)
	C5	100 per cent. H ₂	893.0±28.1	

* EBA = 10 per cent. equine blood agar.

† Colony count expressed as mean±S.E. (standard error of the mean) derived from ten replicate plates, each seeded with 0.02 ml of a 0.5×10^{-3} dilution of the test culture.

Plates were incubated for 48 hr.

‡ t-value for difference of means on 18 degrees of freedom.

strains, ten showed significantly decreased colony counts on plates incubated in H₂ alone. The ultimate recovery of the remaining eight strains was not significantly affected by the absence of CO₂ although the rate of the development of colonies was much slower.

TABLE II

Recovery of Bacteroides strains from dilutions of CMB cultures grown anaerobically on EBA medium in BTL jars with and without carbon dioxide*

Test strain	Jar number	Gas used	Mean colony count±S.E.†	t-value§
18-hr CMB culture of <i>Bacteroides necrophorus</i> no. NCTC7155	C5	10 per cent. CO ₂ +90 per cent. H ₂	739.6±21.2	3.36 (P<0.001)
	C7	100 per cent. H ₂	17.8± 3.5‡	
18-hr CMB culture of <i>B. fragilis</i> no. NCTC9343	C3	10 per cent. CO ₂ +90 per cent. H ₂	1153.0±32.4	6.7 (P<0.001)
	C4	100 per cent. H ₂	873.0±26.4‡	

* EBA = 10 per cent. equine blood agar.

† Colony count expressed as mean±S.E. (standard error of mean) derived from ten replicate plates, each seeded with 0.02 ml of a 0.5×10^{-3} dilution of the test culture.

‡ Plates incubated in 100 per cent. H₂ required prolonged incubation (72 hr) for the development of easily visible colonies; plates incubated in H₂+CO₂ for 18 hr.

§ t-value for difference of means on 18 degrees of freedom.

The growth of five strains of beta-haemolytic streptococci (Lancefield group A) was compared in jars with and without the addition of 10 per cent. CO₂.

Two of the test strains showed significantly improved recovery in the presence of added CO₂ (table III); the colony counts of other strains were not significantly affected. In all cases, however, the size of the colonies, and of the zones of haemolysis, as well as the degree of haemolysis observed, were greater in the presence of CO₂.

The influence of 10 per cent. carbon dioxide on the growth of anaerobic organisms from samples of human faeces

Because *Bacteroides* spp. form a major component of the faecal flora, and the growth of our test strains was enhanced by added CO₂, the yield of anaerobic

TABLE III

Recovery of two strains of Streptococcus pyogenes from dilutions of broth cultures grown anaerobically on EBA medium in BTL anaerobic jars with and without carbon dioxide*

Test culture	Jar number	Gas used	Mean colony count $\pm$ S.E.†	t-value‡
18-hr NB culture of <i>Strept. pyogenes</i> (strain I)	C5	90 per cent. H ₂ + 10 per cent. CO ₂	525.2 $\pm$ 20.3	23.45 (P < 0.001)
	C8			
	C1			
	C7	100 per cent. H ₂	237.4 $\pm$ 5.1	
	C10			
	C12			
18-hr NB culture of <i>Strept. pyogenes</i> (strain IV)	H3	90 per cent. H ₂ + 10 per cent. CO ₂	477.3 $\pm$ 29.5	2.66 (P < 0.05)
	C6			
	H1			
	C11	100 per cent. H ₂	363.1 $\pm$ 31.2	
	H2			
	H4			

* EBA = 10 per cent. equine blood agar.

† Colony count expressed as mean $\pm$ S.E. (standard error of the mean) derived from nine replicate plates, each seeded with 0.02 ml of a 10⁻⁴ dilution of the test organism.

Plates were incubated for 18 hr.

‡ t-value for difference of means on 16 degrees of freedom.

organisms ("total anaerobes") from faecal samples was compared on plates incubated in anaerobic jars with and without the addition of 10 per cent. CO₂. Weighed portions of freshly passed samples of faeces were diluted in pre-steamed nutrient broth and the dilutions were used to seed plates that were then processed as described above. The results of a typical experiment (table IV) show that the colony count of total anaerobes is increased more than three-fold if CO₂ is added to the incubation environment.

Investigation of possible inhibitory effects of carbon dioxide

In the present study there was little evidence of inhibition of growth of strains by CO₂; the test strain of *Cl. histolyticum* showed slight inhibition and

one strain of *Cl. welchii* (C1) showed slight inhibition on one occasion that was not reproducible in further replicate experiments. None of the other *Cl.*

TABLE IV

Yield of anaerobic organisms from a dilution of a sample of human faeces grown anaerobically on EBA medium in BTL jars with and without carbon dioxide*

Jar number	Gas used	Mean colony count $\pm$ S.E.†	t-value‡
C8 } C9 }	90 per cent. H ₂ + 10 per cent. CO ₂	483.0 $\pm$ 7.2	34.35 (P < 0.001)
C6 } H1 }	100 per cent. H ₂	149.1 $\pm$ 6.6	

* EBA = 10 per cent. equine blood agar.

† Each colony count expressed as mean $\pm$ S.E. (standard error of the mean) of 20 replicate plates, each seeded with 0.02 ml of 0.5×10^{-6} dilution of a faecal sample, and incubated for 72 hr.

‡ t-value for difference of means on 38 degrees of freedom.

TABLE V

Recovery of two strains of Cl. welchii from diluted samples of 5-hr CMB cultures grown anaerobically on EBA medium in BTL jars with and without carbon dioxide*

Test culture	Jar number	Gas used	Mean colony count $\pm$ S.E.§	t-value
5-hr CMB culture† of <i>Cl. welchii</i> no. NCTC8359	H3 } C1 }	90 per cent. H ₂ + 10 per cent. CO ₂	437.0 $\pm$ 15.6	1.06 (P > 0.05)
	C8 } C2 }	100 per cent. H ₂	461.9 $\pm$ 17.4	
5-hr CMB culture‡ of <i>Cl. welchii</i> strain L ₂ A	H3 } C1 }	90 per cent. H ₂ + 10 per cent. CO ₂	695.0 $\pm$ 18.6	1.95 (P > 0.05)
	C8 } C2 }	100 per cent. H ₂	632.4 $\pm$ 26.4	

* EBA = 10 per cent. equine blood agar.

† The total cell count was 1.6×10^8 per ml; the spore estimate was $< 3.2 \times 10^5$ per ml (no spores seen in over 500 cells counted).

‡ The total cell count was 1.7×10^8 per ml; the spore estimate was $< 3.4 \times 10^5$ per ml (no spores seen in over 500 cells counted).

§ Colony counts expressed as means $\pm$ S.E. (standard error of the mean) derived from 20 replicate plates, each seeded with 0.02 ml of a 0.25×10^{-3} dilution of the parent culture.

|| t-value for difference of means on 38 degrees of freedom.

welchii strains tested showed any inhibition of growth by CO₂, either when overnight cultures were used, or when young (5-hr) cultures were used in which spores were not abundant (table V). Similarly, no inhibition was found with the Gram-negative anaerobes tested. In jars that contained no H₂ (i.e.,

evacuated and filled with CO₂ alone) so that the atmosphere was predominantly CO₂ but not strictly anaerobic, none of the Gram-negative anaerobes grew; however the *Cl. welchii* strains and one *Cl. bifermentans* strain grew in this atmosphere.

DISCUSSION

The addition of carbon dioxide to the incubation environment in anaerobic jars has often been recommended, yet there is a lack of supporting quantitative data, especially in relation to the yield of anaerobes on solid media. The present study, with statistically designed experiments, has clearly shown that addition of CO₂ in a concentration of 10 per cent. to anaerobic jars increases the recovery on solid media of laboratory strains of clinically important anaerobes and of anaerobes from faecal samples. Concentrations of CO₂ below 10 per cent. were less effective, and concentrations above 10 per cent. did not increase the yield further. Exuberance of growth as measured by colony size and numerical recovery were both increased in the presence of added CO₂. This effect was most noticeable in the case of the Gram-negative anaerobes, but was also found with some test strains of clostridia such as *Cl. tetani*. Just as Holland, Barker and Wolf (1970) noted that the enhancing effect of CO₂ on spore germination varied with different test strains and was influenced by ingredients in the medium, it is reasonable to assume that the effects of CO₂ observed in the present study may be similarly influenced. This requires further investigation.

Anaerobic conditions have been recommended for the isolation of beta-haemolytic streptococci (Jones *et al.*, 1941; Brumfitt and Masters, 1959), and are known to facilitate the development of easily recognisable haemolytic colonies (Fry, 1933). In the present study, the presence of 10 per cent. CO₂ during anaerobic incubation significantly improved the recovery of two strains of haemolytic streptococci. If this finding can be shown to be applicable to other strains it has clear relevance for the diagnostic laboratory.

Although Futter and Richardson (1971) found that recovery from spores of *Cl. welchii* was inhibited by even small amounts of CO₂, no inhibition of growth of these organisms was noted in the present study when essentially vegetative inocula from CMB cultures were grown on blood agar; indeed all of the test strains of *Cl. welchii* grew well in relatively pure CO₂. Similarly, no inhibition of growth of the Gram-negative non-sporing anaerobes was found if 10 per cent. CO₂ was used in the anaerobic atmosphere, although the test strains would not grow in relatively pure "non-anaerobic" CO₂.

It has recently been reported (Ingham *et al.*, 1970) that the addition of CO₂ to the incubation environment has a pronounced effect on the susceptibility of a Gram-negative anaerobe to certain antibiotics *in vitro*, and can therefore affect the performance and interpretation of disk-sensitivity tests. It could be argued, therefore, that the addition of CO₂ to anaerobic jars will lead to misleading results and is not justified. Disk-sensitivity testing is valid, however, only if satisfactory growth (as measured by the number and size of colonies developed) is achieved with reasonable speed; my results suggest that if H₂

alone is used, prolonged incubation is necessary before this can be achieved. The problems attending the use of CO₂ in this context, e.g., pH changes and other factors affecting antibiotic action, must be solved without excluding CO₂ and they merit further study.

The observed growth-enhancing effect of CO₂ for non-sporing anaerobes is of special diagnostic importance in relation to the increasing role of these organisms in serious infections of man (Sencer, 1971; Felner and Dowell, 1971). Thus, the assured provision of CO₂ by the Gaspak system or as a part of a routine anaerobic procedure (see Collee *et al.*, 1972) now merits the attention of the clinical microbiologist when he attempts to isolate clinically important anaerobes on solid media.

SUMMARY

The quantitative recovery of test strains of bacteria on blood agar media was compared in Baird and Tatlock anaerobic jars with and without additional CO₂ in the gas mixture. Both the colony size and the colony count of Gram-negative anaerobes and some of the test strains of clostridia were increased in the presence of added CO₂. An enhancing effect was also noted with two strains of haemolytic streptococci. The addition of 10 per cent. CO₂ as a part of routine anaerobic procedure is recommended for the diagnostic service laboratory.

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