Anaerobic CO₂ Cabinet for the Cultivation of Strict Anaerobes

P. ANN LEACH, J. J. BULLEN, AND I. D. GRANT

National Institute for Medical Research, Mill Hill, London, N.W.7., England

Received for publication 5 August 1971

The design and construction of an anaerobic CO₂ cabinet are described. Air is displaced by a stream of oxygen-free CO₂ and anaerobic conditions are produced in 3 hr. The equipment is simple and cheap to operate and has been found to be satisfactory for the isolation of strict anaerobes from the mouse intestine.

The method devised by Hungate (6) for the cultivation of anaerobic cellulolytic bacteria in an atmosphere of CO₂ is a considerable advance in bacteriological technique, since the entire operation is conducted in the absence of oxygen. However, the necessity for the use of roll tubes instead of petri dishes is a serious disadvantage. In the first place, it is more difficult to distinguish and count different colonies in roll tubes than on plates and more difficult and time-consuming to pick single colonies.

During the past few years, a number of different types of anaerobic cabinets have been described. In every case, the aim has been to provide a relatively large enclosed space entirely free from oxygen in which conventional bacteriological techniques for isolation and subculture can be used. Drasar (4) described a perspex cabinet fitted with glove ports and an airlock. Oxygen was removed by burning a spirit lamp inside the box and removing the last traces of O₂ by pumping the cabinet's atmosphere through alkaline pyrogalloyl or through a deoxygenating column of heated copper. While in use, the cabinet was flushed continuously with oxygen-free nitrogen containing 5% CO₂. The equipment used by Rosebury and Reynolds (9) consisted of a metal glove box fitted with an air lock. A large part of the O₂ was removed by evacuating the box for 8 hr or more, and this was followed by the introduction of 10% H₂ in N₂ which was passed over a palladium catalyst. Aranki et al. (1) used a somewhat similar system to that of Rosebury and Reynolds (9), except that the glove box was made of vinyl plastic and could be collapsed during evacuation. The plastic chamber was attached to a steel air lock which could be rapidly evacuated. The atmosphere within the glove box consisted of 10% H₂ and 5% CO₂ in N₂, and this was circulated by a fan over a palladium catalyst. Lee, Gordon, and Dubos (7) employed a fiber glass glove box which was rendered anaerobic by repeated inflation of a balloon inside the box with nitrogen, the displaced gas being replaced by nitrogen mixed with 5% CO₂. An air lock which could be evacuated and refilled with N₂ was used for the transfer of material into the box. Similar equipment was used by Gordon and Dubos (5), except that the transfer box was rendered anaerobic by passing a stream of N₂ through the chamber for 90 min.

All of these devices are relatively complicated to operate and require the use of an air lock which must also be made anaerobic before the transfer of material into the chamber.

In this paper, we describe an anaerobic cabinet which works on the principle of the displacement of air by a continuous stream of oxygen-free CO₂. Since CO₂ is approximately 1.5 times as dense as air and 1.37 times as dense as oxygen, the production of anaerobic conditions can be achieved quite rapidly. The method is simple and cheap to operate and also has the added advantage that there is no need for an air lock.

MATERIALS AND METHODS

Construction of the cabinet. The cabinet (Fig. 1) was constructed of 17 standard wire gauge [0.056 inch (0.14 cm)] steel plate. The dimensions were 3 ft 6 inches in length, 2 ft 4 inches in depth, and 2 ft in height (1.06 by 0.71 by 0.61 m), with a total volume of 14.1 ft³. The two access ports on the top were made of 0.5-inch (1.27 cm) steel plate and were machined to accommodate a rubber O ring. The access port cover, which rested on the O ring, was of ½-inch (0.79 cm) steel plate and was fitted with a hand grip on top. A gas-tight seal was made by tightening three wing nuts which could be swung onto the top of each cover. The window was made of 0.5-inch perspex sheet and was fastened to the cabinet by a metal frame. The three glove ports projected 2 inches (5.08 cm) from the side of the cabinet and

1 Present address: 31 Bullhead Road, Borehamwood, Hertfordshire.
to wash bottles containing liquid paraffin and from there to the outside atmosphere through tubes inserted in the window frame. The wash bottles acted as traps to prevent any admission of air if the CO₂ supply was turned off; they also acted as safety devices since the bubbling of the CO₂ through paraffin oil could be readily seen and this ensured that the gas was passing to the outside of the building.

Ancillary equipment: Semiautomatic electric sterilizer. This was designed for the sterilization of the tops of 1-oz universal bottles, containing liquid cultures (Fig. 3). The heating element consisted of two 11-ohm lengths of 24 standard wire gauge nichrome resistance wire connected in parallel. This was mounted inside a block of Asbestolux which was machined to accommodate the neck of the bottle. Power was provided from the 14-v secondary winding of a transformer. The heated wire gave a temperature of about 450°C after a short warm-up period. The neck of the bottle was sterilized by a heating period of 12 sec which was controlled by a synchronous motor and a microswitch. A neon light was switched on when heating was started and went out when sterilization was complete. The spring-loaded platform automatically held the bottle in position. This arrangement also had the advantage that the bottle could be safely left on the platform after sterilization, the asbestolux block acting as a sterile cover.

Operation and performance. Both liquid media in bottles and solid media in petri dishes were placed in anaerobic jars (Baird and Tatlock Ltd.) for 18 to 24 hr before use. All of the kinds of equipment needed, such as pipettes, diluting fluid, syringes, sterile platinum loops, glass homogenizers, and other

![Figure 1. Diagram of anaerobic cabinet. (1) CO₂ cylinder, (2) infrared lamp, (3) flowmeter, (4) furnace, (5) gas inlet tubes, (6) glove ports, (7) entry port, (8) manometer, (9) fluorescent lamp, (10) gas exit tubes, (11) wash bottles, (12) laboratory window frame, (13) outside atmosphere, (14) cabinet stand.](image)

![Figure 2. PO₂ of effluent gas from cabinet. Flow rate was 6 liters of CO₂ per min.](image)
instruments, were placed in anaerobic jars and left 18 to 24 hr. Tubes containing a few grams of CaCl₂ were placed in jars containing plates to prevent the development of excessive moisture on the agar surface. Next day the jars were placed inside the cabinet together with 20 ml of resazurin indicator in an open universal bottle. The indicator consisted of the basal medium of Mann (8), except that the rumen fluid was replaced by water and 0.5% glucose was added. The flow of oxygen-free CO₂ was adjusted to 6 liters/min. The resazurin indicator became colorless after 1.5 hr. For routine work, the CO₂ was run for 3 hr at 6 liters/min and for the remainder of the time at 3 liters/min. After inoculation, all plates were placed in anaerobic jars within the cabinet, whereas liquid cultures were either placed in jars or sealed with screw caps. The anaerobic jars were removed from the cabinet, evacuated on a water pump until about 5% of CO₂ remained, and then were filled with H₂ in the usual way. Sealed liquid cultures were placed in an ordinary incubator.

In one test, the PO₂ in the cabinet was measured with an oxygen electrode by passing the exit gas through the equipment described by Bullen, Rogers, and Lewin (3). In this case, a second resazurin indicator was placed near the top of the cabinet. This was reduced by 2.5 hr. The test showed that the exit gas contained no oxygen after 3 hr (PO₂ < 1.0 mm of Hg; Fig. 2). When the cabinet was anaerobic, material such as mouse intestine could be introduced through one of the top ports without greatly disturbing the anaerobic conditions. When this was done, the CO₂ was left running at 6 liters/min. In the test described, opening a top port for the few seconds required to introduce a sample allowed the PO₂ in the exit gas to rise to 8 mm of Hg. This was reduced to zero in 20 min. Neither of the resazurin indicators became oxidized (E’o of resazurin is −42 mv, pH 6.9; reference 10). In routine work, the mouse intestine was introduced into the cabinet in a universal container already filled with CO₂, and none of the anaerobic jars containing media was opened until an additional 20 min had elapsed.

Further tests showed that N₂ could not be used instead of CO₂. With 6 liters of oxygen-free N₂ per min, the resazurin indicator remained oxidized for at least 8 hr.

RESULTS

In this paper, no attempt is made to discuss the bacteriology of the mouse intestine, but it is perhaps worth recording the isolation of the strictly anaerobic "tapered rods" or "fusiform" bacteria that have been described by Gordon and Dubos (5). It has been the experience of these authors, and also our own, that it is impossible to cultivate these bacteria on the surface of agar plates without the aid of an anaerobic cabinet. Thus far, we have obtained over 50 cultures of "fusiform rods" from mouse cecal contents. The most suitable medium for surface growth has been found to be B.G.P. maintenance medium (2) with the addition of 1.0% CO₂. The organisms also grew on the surface of blood-agar.

Within the anaerobic cabinet, these bacteria can be grown indefinitely both on the surface of solid medium and in liquid cultures. Attempts at culture outside the cabinet showed that very heavy inocula of liquid cultures into fresh liquid medium containing a reducing agent (B.G.P. broth) gave satisfactory growth, but all attempts to grow the organisms on the surface of agar plates were unsuccessful, even when the plates were transferred to an anaerobic jar within seconds of plating out.

DISCUSSION

There is little doubt that anaerobic cabinets can be extremely useful for the surface cultivation of certain strict anaerobes (5; see above). The anaerobic cabinets described previously depend upon the removal of O₂ by a variety of different means. In some, the last traces of oxygen are removed by a catalytic reaction with H₂ or by other chemical means (1, 4, 9). Others depend upon vacuum pumps or the displacement of air by inflation of an internal balloon (7). All require an air lock which must be rendered anaerobic by evacuation or some other means before transfer of material into the cabinet.

The use of CO₂ in a large rigid box seems to have a number of advantages. The cabinet is simple to operate, a supply of oxygen-free CO₂ being all that is required. At a flow rate of 6 liters/min, the cabinet becomes anaerobic in 3 hr. The simplicity of operation ensures that anaerobic conditions can be reproduced without fail, and there is no need to worry about the efficiency of catalysts or vacuum pumping systems. The cabinet is sufficiently large (14.1 ft³) to accommodate large amounts of equipment. The density of the CO₂ is such that briefly opening one of the top

---

Fig. 3. Semiautomatic electric sterilizer for Universal bottles.
ports for the introduction of material does not affect the anaerobic indicators, and tests have shown that the exit gas becomes free from oxygen after an additional 20 min. The equipment is cheap to operate, the cost of CO₂ being approximately 38¢ per run. There is a high degree of safety in operation, provided that the wash bottles are inspected occasionally to ensure that the gas is passing to the outside atmosphere. No difficulties have been encountered in keeping pure cultures on agar plates but it is essential to sterilize the neck of liquid culture bottles to avoid contamination, and the electrical sterilizer described has proved to be invaluable for this purpose.

ACKNOWLEDGMENTS

Thanks are due to W. C. Lister, K. Green, R. B. Bower, and F. New, National Institute for Medical Research, for help with the design and for construction of the cabinet, to G. Finch for the design and construction of the electrical sterilizer, and to F. New for Fig. 1. We thank the Photography Section, National Institute for Medical Research, for Fig. 3.

LITERATURE CITED

1. Aranki, A., S. A. Syed, E. B. Kenney, and R. Freter. 1969. Isolation of anaerobic bacteria from human gingiva and mouse caecum by means of a simplified glove box procedure. Appl. Microbiol. 17:568–576.
2. Beerens, H. 1954. Amélioration des techniques d’étude et d’identification des bactéries anaérobies. Ann. Inst. Pasteur Lille 6:36.
3. Bullen, J. J., H. J. Rogers, and J. E. Lewin. 1971. The bacteriostatic effect of serum on Pasteurella septica and its abolition by iron compounds. Immunology 20:391–406.
4. Drasar, B. S. 1967. Cultivation of anaerobic intestinal bacteria. J. Pathol. Bacteriol. 94:417–429.
5. Gordon, J. H., and R. Dubos. 1970. The anaerobic bacterial flora of the mouse caecum. J. Exp. Med. 132:251–260.
6. Hungate, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. 14:1–49.
7. Lee, A., J. Gordon, and R. Dubos. 1968. Enumeration of the oxygen sensitive bacteria usually present in the intestine of healthy mice. Nature (London) 220:1137–1139.
8. Mann, S. O. 1968. An improved method for determining cellulolytic activity of anaerobic bacteria. J. Appl. Bacteriol. 31:241–244.
9. Rosebury, T., and J. B. Reynolds. 1964. Continuous anaerobiosis for cultivation of spirochetes. Proc. Soc. Exp. Biol. Med. 117:813–815.
10. Twigg, R. S. 1945. Oxidation-reduction aspects of resazurin. Nature (London) 155:401.