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Atmospheric Analysis and Redox Potentials of Culture Media in the GasPak System

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Oxygen and carbon dioxide concentrations, internal atmospheric pressure, catalyst temperature, and time of appearance of water condensate were monitored for various time intervals at ambient (20 to 25°C) temperature in a GasPak 100 Anaerobic System (BBL Microbiology Systems, Cockeysville, Md.). Simultaneously, the redox potential (Eh) of various plated culture media in the system was also measured. The oxygen concentration was reduced to less than 0.4% in 100 min. The Eh of the media, corrected for hydrogen ion, reached −100 mV within 60 to 100 min, and the carbon dioxide concentration increased to between 4 and 7% in 60 min, depending on the number of plates of media present. Condensate appeared generally between 10 and 15 min, and the temperature of the lid reached a maximum between 20 and 40 min. Condensate time and lid temperature increase are important early indicators of a correctly functioning GasPak System. A characteristic pressure-vacuum-pressure profile is produced as a result of controlled release of hydrogen and carbon dioxide gases and the reaction of hydrogen and oxygen to produce water. Anaerobic conditions were achieved well before the methylene blue anaerobic indicator became decolorized, which required more than 6 h at 20 to 25°C. At this time the Eh of media in the jar was well below −200 mV. Since the indicator is reduced within 5 h at 35°C, the Eh of media in the jar would also be expected to decrease more rapidly at the higher temperature.

A number of investigators (13, 22, 26) have reported changes in the oxidation-reduction potential (Eh) of media during the culture of various bacteria, although there are relatively few reports of this parameter in sterile media. Previous reports on atmospheric conditions in the GasPak System (BBL Microbiology Systems, Cockeysville, Md.) have been those of Brewer and Allgeier (5), Collee et al. (9), and Ferguson et al. (12).

It is often cited that a minimum requirement for growth of anaerobic bacteria is an Eh of −100 mV (2, 19, 20). An anaerobic indicator, usually methylene blue or resazurin, is used to determine whether an anaerobic system is functioning properly (6). The Eh for methylene blue of +11 mV, quoted in the literature (8, 18), is considered inadequate. However, this is the Eh at 50% reduction and by no means indicates the Eh of the colorless state. Recently, it was reported in the *VPI Anaerobe Manual* that the Eh of methylene blue indicator when colorless (99.9% reduced) is −230 mV (14).

Since the initial report of Brewer and Allgeier in 1965 (4), there have been a number of improvements in the design of the GasPak System. There has not been any published information on the System as it now exists. The purpose of this investigation is to provide an analysis of atmospheric conditions within an activated GasPak System and to correlate these with the Eh of culture media contained in the jar, the time of appearance of water condensate, lid temperature changes, and color changes of the anaerobic indicator.

(This work was presented, in part, at the 76th Annual Meeting of the American Society for Microbiology, Atlantic City, N.J., 1976.)

MATERIALS AND METHODS

Media. The following media were employed: Trypticase soy agar with 5% sheep blood (TSA II), Columbia agar with 5% sheep blood, and Schaedler agar with vitamin K_1 and 5% sheep blood. All were commercially prepared (BBL Microbiology Systems), plated media, stored under refrigeration and brought to room temperature before use. Analytical tests described in this paper were conducted within 2 weeks of plate manufacture.

Anaerobic equipment. A complete GasPak 100 System (Fig. 1) was used, consisting of the following: GasPak hydrogen- and carbon dioxide-generating envelopes; GasPak polycarbonate anaerobic jar; disposable methylene blue indicator (6); catalyst chambers containing 2.5 ± 0.5 g of palladium catalyst pellets; and a metal anaerobic jar lid with holes for the pressure, oxygen, and Eh detectors (Fig. 2). The system was tested for leaks before each experiment.

Temperature. Temperature changes of the catalyst were indirectly determined by measuring the tem-

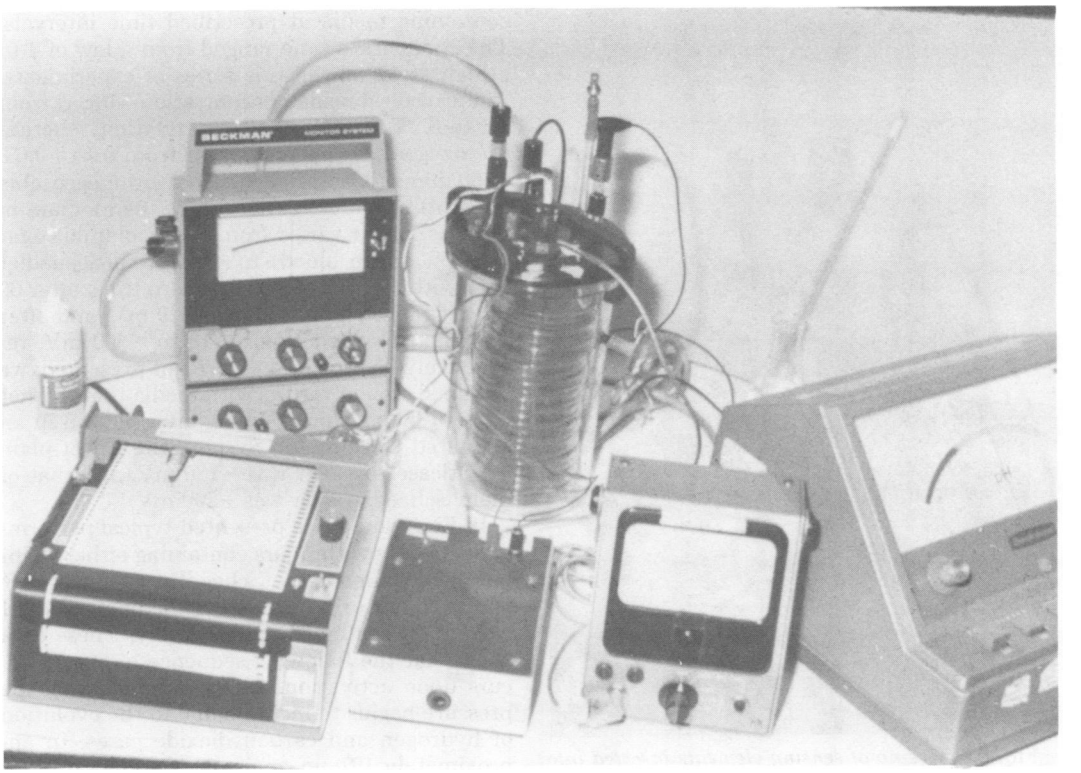

FIG. 1. *Instruments and sensing elements used to monitor atmospheric conditions, temperature, and Eh of culture media in the GasPak System. Front row, left to right, are: pressure recorder, pressure response amplifier, temperature meter, potentiometer (pH meter); back row, left to right, are: oxygen analyzer and anaerobe jar with sensing elements in the lid.*

perature of the lid using a Yellow Springs model 42SC Tele-Thermometer employing a YSI 423 thermocouple probe. The tip of the probe was inserted into a cavity drilled into the center of a Brewer anaerobic jar metal lid just above the catalyst chamber site.

Pressure changes. The pressure within the GasPak 100 jar relative to atmospheric pressure was measured with a Kulite semiconductor type ITQ500E pressure transducer inserted through a hole in the jar lid by means of a rubber bung and connected to a Data Technology Gould Brush 220 recorder set at a 1-mV sensitivity (Fig. 1). The recorder was calibrated so that a 1-in. (ca. 2.54-cm) deflection of the recorder pen, either in the positive or negative direction, was equivalent to a pressure change of 1 lb/in^2.

Oxygen concentration. Oxygen concentration was measured by means of a Beckman 586045 oxygen probe inserted through the jar lid by means of a rubber bung and connected to a Beckman model 123305 Monitor System. The electrode was standardized at atmospheric pressure (1 Torr) and against the normal oxygen concentration of air (20.9%).

Carbon dioxide concentrations. Internal atmospheric samples were withdrawn by means of a needle and syringe inserted through a clamped rubber tube attached to ports at the side of the GasPak anaerobe jar. A Hewlett-Packard model 7530A gas chromatograph, equipped with dual Porapak 80-to-100-mesh columns, each 6 ft (ca. 1.83 m) in length and 0.125 in (ca. 0.32 cm) in diameter, was used to determine carbon dioxide concentration. Helium was used as the carrier gas, and the flow was adjusted to 35 ml/min at 60 lb/in^2. The thermal detector was set at 150°C. A standard curve was established by volumetric withdrawal and addition of known volumes of pure carbon dioxide gas to a GasPak 100 jar of 2,500-ml volume. The attenuation of the gas chromatograph was set at 32, the sensitivity was set at 5, the chart drive was 4 in. (ca. 10.16 cm) per min, and the oven was programmed to maintain 25°C.

Redox potential. The Eh of culture media was measured using published procedures (1, 21, 26, 29). In most cases the jar was filled with empty petri dishes except for the uppermost dish. For comparative purposes, some measurements were made with a full stack of 11 dishes of the same medium. With the lid removed, the uppermost dish was positioned so that a polished platinum thimble electrode (Beckman no. 39271) and a Beckman calomel reference electrode (Beckman no. 39400), fitted into the lid of the jar by means of a rubber bung (Fig. 2), would penetrate the surface of the medium. The electrodes were attached to a Beckman model 76 potentiometer (pH meter) set for millivolt measurements and zeroed using the Beckman PT 8980 shorting strap. For standardization, a saturated solution containing approximately 200 mg of

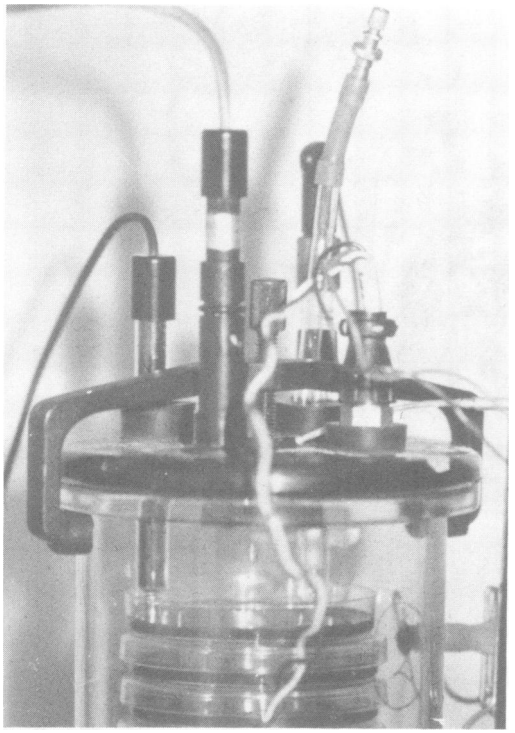

FIG. 2. *Close-up of sensing elements inserted into the lid of the anaerobic jar.*

quinhydrone (Baker no. U755) in 100 ml of pH 4 phthalate buffer (Arthur H. Thomas Co., Philadelphia, Pa., catalog no. 4120-G12) was used. The observed millivolt reading of the standard at 20 to 25°C was within ±6 mV of the published value of +218 mV (21, 29).

The following formula was used to convert observed potentiometer readings in millivolts (E) to the corrected redox potential (29): Eh = E + 244 mV.

Experiments had shown that the temperature of the medium in the jar being monitored did not increase more than 2°C. Similar to the findings of Onderdonk et al. (21), the pH did not change more than 0.3 units. Therefore, corrections for pH and temperature in the Eh calculation were not considered necessary (1, 29).

A series of experiments were also conducted by adding oxygen in the form of atmospheric air in various increments and measuring the Eh of the culture medium in the jar immediately after each addition.

RESULTS

Performance profile data of the GasPak H_2 and CO_2 gas-generating envelope for various physical and chemical parameters are presented in Tables 1 and 2 and in Fig. 3 to 5. In Table 1, data are presented for time of appearance of water droplets (condensate time), maximum lid temperature, carbon dioxide concentration, oxygen concentration, and Eh of various blood-containing media at prescribed time intervals. The condensate time ranged from a low of 4 to a high of 12 min in this series of experiments. The carbon dioxide concentration ranged from 4.6 to 6.2% at 60 min after activation, whereas the oxygen concentration was from 0.2 to 0.6% at 60 min and less than 0.2% (lowest measurable amount) at 100 min. The Eh of the medium in the GasPak jar varied from +60 (Columbia agar with 5% sheep blood) to +400 mV (Schaedler agar with 5% sheep blood) at zero time; after 60 min, it ranged from −30 to −229 mV, and after 100 min it ranged from −115 to −300 mV, respectively. Although data are not presented, we obtained similar results with media that did not contain blood. For example, after 60 min in an activated GasPak 100 System, the Eh of plain Trypticase soy agar was −130 mV, and that of plain Schaedler agar was −200 mV.

In Fig. 3 and 4 are presented typical performance profiles within jars containing either a single plate of medium (Schaedler agar with 5% sheep blood) or a full load of medium (TSA II with 5% sheep blood), respectively. These data show that the following sequence of events occurs upon activation. Almost immediately the pressure begins to increase due to the evolution of hydrogen and carbon dioxide gases. In approximately 10 min, moisture (condensate) appears on the wall of the jar, signaling the formation of water as a result of the catalyzed reaction of hydrogen and oxygen gases. At this point, the oxygen concentration is seen to fall precipitously while the lid temperature increases due to the reaction heat generated by the palladium catalyst. Shortly after the appearance of condensate, the pressure reaches the first peak and then begins to decrease and becomes negative relative to the outside atmospheric pressure (vacuum phase), due to the removal of oxygen. As carbon dioxide and hydrogen continue to be produced, the pressure becomes positive again and increases until a plateau is reached at about 2 to 3 lb/in^2. The CO_2 concentration generally reaches a maximum of 5 to 7% in approximately 60 min. The Eh of the culture medium reduces rapidly or slowly, depending on the type of culture medium and the number of plates of medium in the jar. The Eh becomes a negative value near the end of the vacuum phase or shortly thereafter, usually reaching −100 mV within 60 to 90 min.

The effect of the number of plates on the timing of various events in the performance profile can be seen in Fig. 3 and 4, and also in Fig. 5, which shows the performance profile in jars containing 1 plate versus 11 plates of the same medium (Columbia sheep blood agar). The data for Fig. 5 for certain events are tabulated in

TABLE 1. *Performance profile data in the GasPak System at various time intervals*

Medium	Condensate time (min)	Lid temp maximum (°C)	% CO_2 at 60 min	% O_2 at:		Eh (mV) at:		
				60 min	100 min	0	60 min	100 min
Schaedler agar with 5% sheep blood	12	33	6.2	0.3	0.2	+400	−229	−300
	7	35	6.0	<0.2	0.2	+340	ND[a]	−195
	9	30	6.0	0.6	<0.2	+378	−30	−115
	6	31	4.6	0.3	<0.2	+245	−150	ND
	8	32	5.2	0.3	<0.2	+90	−130	−200
	4	31	ND	ND	ND	+253	−188	−216
Trypticase soy agar with 5% sheep blood (TSA II)	8	ND	4.3	<0.2	<0.2	+175	−181	−230
	6	34	4.1	<0.2	<0.2	+200	−170	−200
	11	31	6.2	0.6	<0.2	+165	ND	−140
	12	35	5.6	0.4	<0.2	+250	ND	−160
Columbia agar with 5% sheep blood	9	33	4.7	0.4	0.3	+130	−100	−200
	9	31	4.7	0.5	0.4	+60	ND	−150
	7	34	6.5	0.7	0.4	+144	−100	−271

[a] ND, Not determined.

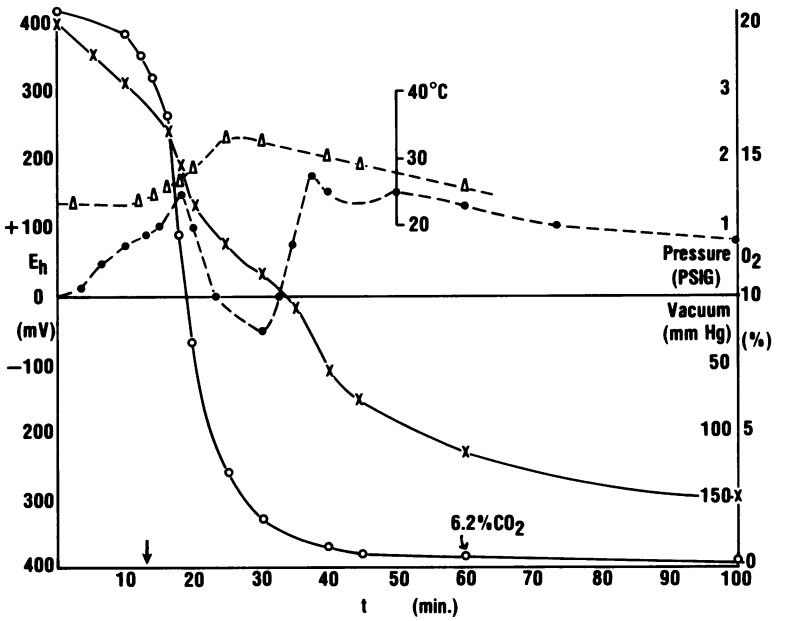

FIG. 3. *Performance profile of a GasPak H_2 + CO_2 envelope in a GasPak 100 System containing a single plate of Schaedler agar with 5% sheep blood. Symbols:* (○) *oxygen concentration;* (●) *pressure (lb/in^2);* (×) *Eh;* (△) *lid temperature. Arrow on abscissa indicates condensate time.*

Tables 2 and 3. The effect on oxygen consumption, lid temperature, and condensate time appears to be minimal. However, the second pressure peak appears to be delayed, as is the time of maximum carbon dioxide concentration and minimum Eh values. The carbon dioxide concentration was 1.0 to 1.5% less with a full complement of plated media, but the time of peak concentration was not affected (Table 3).

A series of experiments were conducted to characterize profile events in relation to the color of the methylene blue anaerobic indicator. At ambient temperature, the indicator rarely becomes completely decolorized before 6 h, whereas at 35°C it is usually decolorized in less than 5 h. Since previous experiments showed that an Eh in the medium of −100 mV is generally achieved in less than 2 h, the Eh at the time of complete reduction of the indicator was determined.

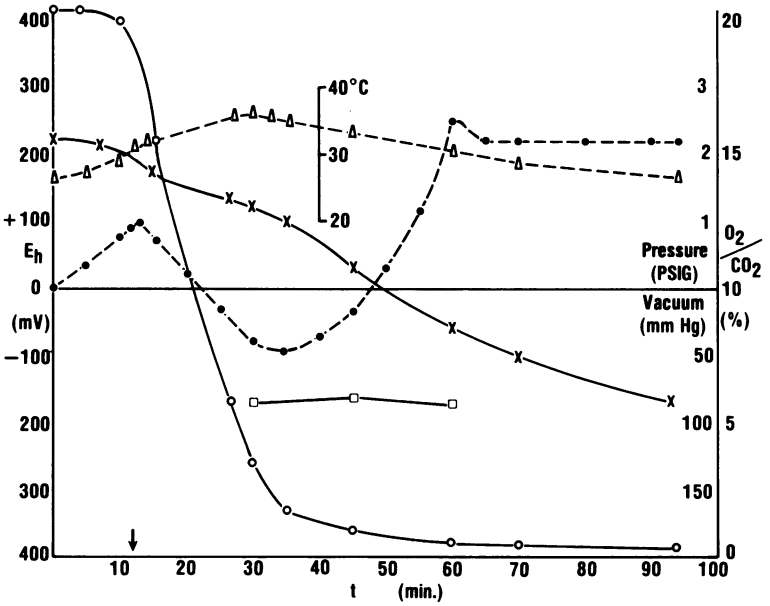

FIG. 4. *Performance profile of GasPak H_2 + CO_2 envelope in a GasPak 100 System filled with 11 plates of Trypticase soy agar with 5% sheep blood (TSA II). Symbols:* (○) *oxygen concentration;* (●) *pressure (lb/in²);* (×) *Eh;* (□) *carbon dioxide concentration;* (△) *lid temperature. Arrow on abscissa indicates condensate time.*

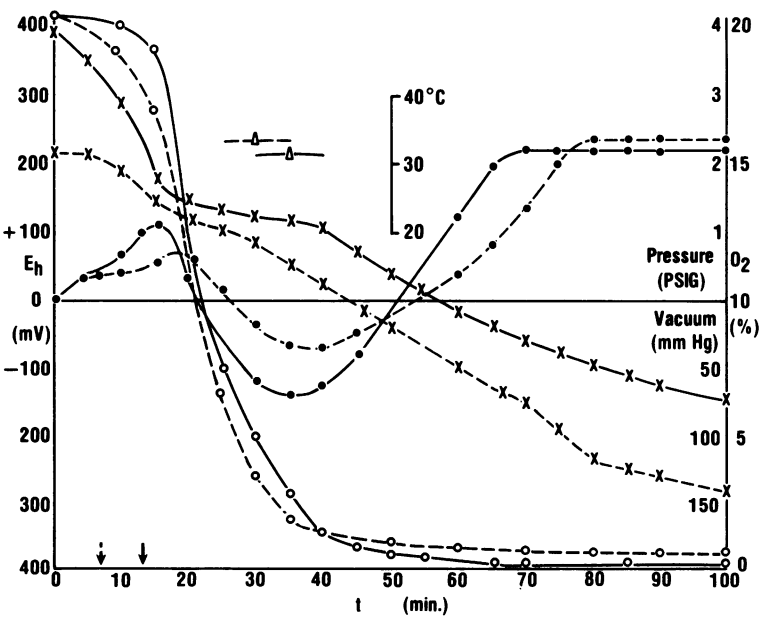

FIG. 5. *Effect of quantity of plated media (Columbia agar with 5% sheep blood) on Eh, pressure oxygen concentration, and carbon dioxide concentration in the GasPak 100 System. Solid lines, 11 plates; broken lines, 1 plate. Symbols:* (○) *oxygen concentration;* (●) *pressure (lb/in²);* (×) *Eh;* (△) *lid temperature. Arrow on abscissa indicates condensate time.*

In most experiments it was not possible to observe the precise moment of decolorization of the indicator. The Eh of media in the GasPak jar generally reached between −250 and −300 mV before the indicator was decolorized. After overnight incubation at 20 to 25°C, the indicator was always completely decolorized, and the Eh of culture media was generally between −300 and −400 mV.

The effect of addition of air on the Eh of a

TABLE 2. *Effect of the number of plates of Columbia agar with 5% sheep blood on various events in the GasPak 100 System*

Event	Time of event (min)	
	1 plate	11 plates
Condensate time[a]	7	13
Maximum carbon dioxide concentration	16	30
Maximum lid temperature	30	35
Minimum oxygen concentration	50	45
Eh (reduced to −100 mV)	61	85

[a] Condensate time is the time moisture is observed to form on the inside walls of the anaerobic jar.

TABLE 3. *Effect of quantity of plates of medium (Columbia agar with 5% sheep blood) on carbon dioxide concentration in the GasPak 100 System*

Time (min)	% Carbon dioxide	
	1 plate	11 plates
15	5.1	4.2
30	6.8	5.7
45	6.5	5.5
60	6.5	5.0
75	5.7	4.2
100	5.0	4.0

culture medium in the jar after anaerobic conditions had been achieved is shown in Fig. 6. The Eh of TSA II plates increased in proportion to the amount of air added to the jar, increasing from −106 mV to −66 mV with the addition of 350 ml of air (2.8% oxygen in the 2,500-ml jar).

DISCUSSION

Performance profile data for a number of physical and chemical parameters of the present-day GasPak hydrogen- and carbon dioxide-generating envelope in a GasPak 100 System at ambient temperature (25°C) are presented.

Loesche reported that anaerobes fall into three categories: strict, which grow maximally at an oxygen concentration of less than 0.5%; moderate, tolerating up to 3% oxygen; and microaerophilic, which grow preferably between 0.3 and 20% oxygen (17). Other reports confirmed that anaerobic organisms have varying degrees of aerotolerance, with some requiring very low redox potentials for optimal growth (17) and others being fairly aerotolerant (17, 20, 21, 23).

The use of fresh media is considered a requisite for good anaerobic culture technique (30). However, a recent report indicates that all of 10 anaerobes grew well on media stored in air for 6 weeks (P. R. Murray and J. L. Christman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, C201, p. 343).

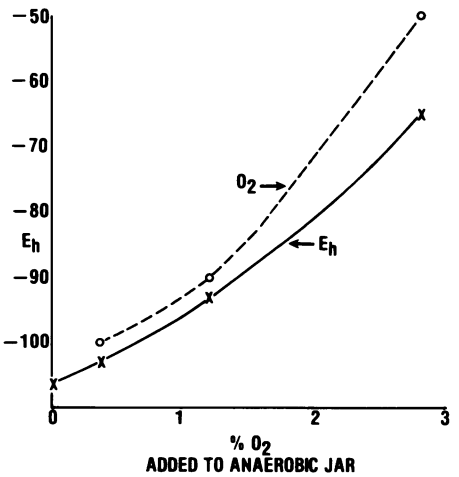

FIG. 6. *Effect of adding oxygen in the form of air to the anaerobic jar on the Eh of media in the GasPak system.*

It is recognized that the absorption of oxygen by culture media may lead to the formation of toxic components such as peroxides (7). Walden and Hentges (27) have shown that oxygen concentration is more important than Eh. Although oxygen concentration may be more important, the minimum requirement usually cited for the culture of anaerobes is a redox potential (Eh) of −100 mV (2, 19, 20). In the GasPak System, oxygen was observed to decrease to less than 0.4% within 100 min, and the Eh of the culture media was reduced to −100 mV between 1 and 2 h at 25°C, depending on the volume of media in the jar.

The pressure-vacuum-pressure cycle characteristic of the GasPak hydrogen- and carbon dioxide-generating envelope is due to the controlled evolution of the gases because of its unique design. Water in the reservoir side of the envelope passes through a piece of filter paper to the retort side, first reaching the borohydride tablet (at the bottom), causing the release of hydrogen. Within a few minutes, water begins to reach the upper carbon dioxide-generating tablet.

The controlled release of hydrogen prevents venting of this gas to the outside and creates a vacuum phase that aids in drawing dissolved oxygen from the media in the jar. The carbon dioxide and excess hydrogen being generated then return the atmosphere to a positive pressure, as can be seen in the profile curves.

A previously published report described the prereduction in the GasPak System of culture media with formulations modified to be reducible (10). An important finding of this study is the demonstration that commonly used culture

media can be reduced quite readily in the GasPak System. The results reported here indicate that this can be achieved within 2 h.

It was observed that the methylene blue indicator did not become decolorized in less than 6 h at 25°C even when the Eh of media in the jar was well below −200 mV. However, color change occurs rapidly in the reverse direction (oxidation), since a trace amount of oxidized dye becomes visible. These results compare favorably with the recently published −230 mV Eh value of methylene blue indicator when 99.9% reduced (14). At 35°C, the methylene blue indicator becomes decolorized more rapidly, usually within 5 h, and one would predict that, likewise, the Eh of culture media within the jar would decrease more rapidly than at 25°C. Thus, although relatively slow in indicating achievement of anaerobiosis, methylene blue is a quite reliable indicator for this purpose.

Condensate (moisture) formation and warming of the anaerobe jar lid are important early indicators of a properly functioning GasPak System and can be reliably used for quality control. The condensate time should be between 2 and 25 min; it was observed to be generally between 7 and 15 min. Maximum temperature of the lid occurs between 20 and 40 min and indicates properly functioning catalyst.

A number of investigators have indicated that carbon dioxide is required by certain anaerobes or that their growth is stimulated by it (20, 24, 28, 30). A 10% CO_2 requirement was claimed by Watt (28), whereas a 5% optimum level was reported by Stalons et al. (24). Results previously reported from our laboratories suggested a minimum requirement of 4% CO_2 for optimum growth of certain anaerobes. It was also found to be required only during the first 4 h of incubation (J. Abramson, P. Bathurst, and G. L. Evans, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, C90, p. 41).

The CO_2 concentration, as measured by gas chromatography, was observed at 60 min after activation to be between 5.0 and 6.8% with one plate, and 4.0 to 5.7% with a full stack of 11 plates (Table 3). Since one would expect a higher concentration due to the decreased internal volume with a full complement of plated media, it is concluded that the decreased CO_2 concentration is due to absorption by the medium. The absorbed CO_2 may still provide the stimulatory effect on growth even though the atmospheric content appears less. The volume of CO_2 (250 ml) generated by a GasPak H_2 + CO_2 envelope is calculated to produce a 10% concentration, but this is probably not achievable, due to absorption of CO_2 not only by media in the jar, but also by the alkaline residue of the borohydride tablet (12). However, one must consider the effect of excessive CO_2 on antibiotic activity when performing susceptibility tests (11), or on pH when biochemical identification tests for anaerobes are performed (24).

In conclusion, the GasPak System has been shown to be a reliable and convenient method of producing an anaerobic environment. Its widespread use and satisfactory performance have been described in numerous publications (3, 10, 15, 16, 25).

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