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pH and Pressure in Closed Tissue Culture Vessels

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When using closed vessels (i.e. with tight fitting closures) for cell culture, one faces certain disadvantages which do not exist for open vessels (i.e. with loose fitting closures) incubated in an appropriate atmosphere.

The disadvantages, concerning pH and pressure, are due to instability of the atmosphere in a closed system. On the other hand, open culture vessels also have disadvantages such as evaporation, which was discussed in Thermo Scientific Nunc Bulletin No. 2.

Table 1 summarizes the advantages and disadvantages of both kinds of vessels.

	Closed vessel	Open vessel
pH	Variable	Constant
Pressure	Variable	Constant
CO_2 gassing	Manual	Automatic
Evaporation	No	Yes
Contam.risk	Relatively low	Relatively high
Incubator	Thermostat only (inexpensive)	Thermostat + CO_2/hum. controls (expensive)

Table 1
Advantages and disadvantages (red) of open and closed culture vessels.

pH

For the control of pH in in vitro cell culture, one simulates the conditions found in blood. One of the main buffer systems in blood is based on the bicarbonate ion, HCO_3^-, as illustrated in Fig. 1.

This means that the blood HCO_3^-, and thus the pH (i.e. the negative logarithm of the H^+ concentration), is in equilibrium with the CO_2 in the lungs. In the natural system, a blood HCO_3^- concentration of 24 mm (corresponding to about 2 mg $NaHCO_3$ per mL) is in equilibrium with a lung CO_2 partial pressure of 40 mm Hg (corresponding to about 5% CO_2) implying the physiological pH 7.4.

Hence, these conditions are adopted for in vitro cell culture by adding 2 mg $NaHCO_3$ per mL of medium and maintaining an atmosphere of 5% CO_2 above the medium.

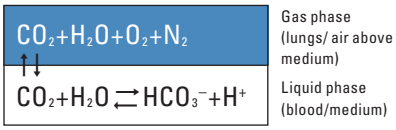

Fig. 1
The bicarbonate buffer system. Note that the buffer equilibrium in the liquid phase is dependent on the CO_2 in the gas phase due to exchange between gaseous and dissolved CO_2.

Some cell types, however, may prefer higher or lower bicarbonate concentrations in the medium. This requires higher or lower CO_2 concentrations above the medium.

In order to maintain a physiological pH according to the Henderson-Hasselbalch equation which may be written as follows:

$$pH = 6.1 + \log\left(52\frac{mg/mL\ NaHCO_3}{\%CO_2} - 1\right).$$

The corresponding values of $NaHCO_3$ concentration, CO_2 concentration, and pH, satisfying this equation, can be estimated from Fig. 2.

Three frequently used media, representing a wide CO_2 range, are Eagle's minimal essential medium (MEM) with Earle's salt solution, MEM with Hank's salt solution, and Dulbecco's modified MEM, whose bicarbonate-CO_2 relationships are given in Table 2.

In open culture vessels, physiological pH is maintained by incubation in a properly adjusted CO_2 incubator.

In closed culture vessels, however, the pH is dependent on the initial pH, the free air space in the vessel, and the respiration of the cells.

The dependence on the initial pH and the air space is demonstrated by the experimental results shown in Fig. 3.

It appears that for each particular air/medium volume ratio the pH increment, due to CO_2 escape to the air space in the vessel, remains constant for initial pH values up to 7.5. Above this value, the increments decrease towards zero.

For initial pH values up to 7.5, the final pH values can be predicted with good accuracy by the following "semi-empirical" formula:

$$pH_{FINAL} = pH_{INITIAL} + 1.25\frac{A}{A+M}$$

where A and M are the air and medium volumes respectively in the vessel. The formula implies that pH_{FINAL} equals $pH_{INITIAL}$ for A = 0.

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	$NaHCO_3$		CO_2
	mm	mg/mL	%
MEM w. Hank's salts	4	0.35	1
MEM w. Earle's salts	24	2.0	5
Dulbecco's mod. MEM	44	3.7	10

Table 2
Corresponding physiological standard values of bicarbonate and CO_2 concentrations, obtained from Fig. 2, for variants of Eagle's minimal essential medium (MEM).

The formula also assumes that the pH increments are independent of the initial bicarbonate concentration, which is demonstrated by the results in Fig. 4.

Table 3 shows the relationships between measured pH increments and those calculated according to the formula.

The cells' CO_2 production through respiration does not influence the initial rise in pH, which is mainly due to the distribution of dissolved CO_2 between the liquid and the gas phase in the vessel. From the results in Fig. 5, it can be seen that this process is a rather abrupt one, as the main pH change has already occurred within the first hour of incubation, when the cells have not yet produced significant amounts of CO_2. Thus, the influence of the cells respiration is a long term effect which becomes apparent only after days of incubation.

However, the ability of cells to restore the optimal CO_2 level is strongly dependent on the bicarbonate concentration used (Table 2). Thus, the restoration may be relatively rapid using MEM with Hank's salts, which only requires 1% CO_2 of which the atmosphere a priori contributes with its 0.03%. This medium would often be chosen for primary cells, because usually their metabolic activity (respiration) is low.

The CO_2 restoration may be left to the cells when using MEM with Hank's salts, whereas the use of more strongly buffered media normally requires gassing of the

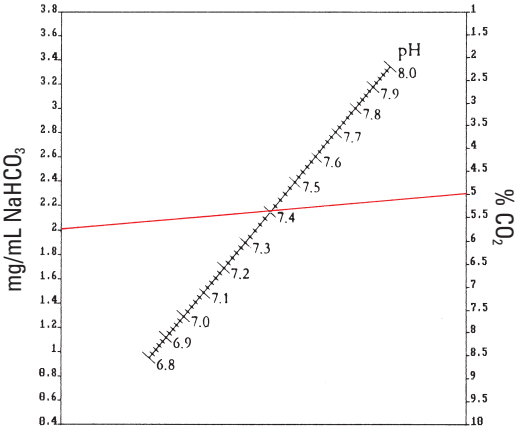

Fig. 2
Nomogram for estimation of corresponding $NaHCO_3$, CO_2, and pH values according to the Henderson-Hasselbalch equation given in the text. The red interpolation indicates the physiological standard values for Eagle's minimal essential medium with Earle's salts.

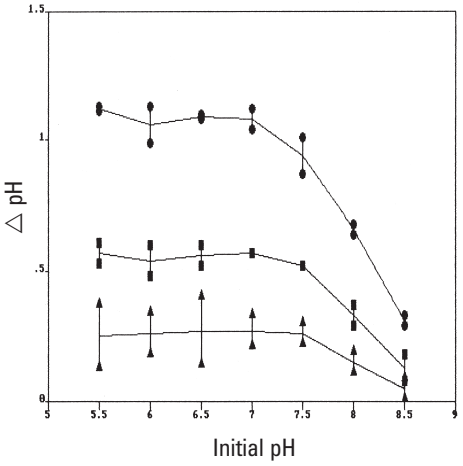

Fig. 3
Measured pH increments, △ pH, in 30 mL (●), 130 mL (■), and 230 mL (▲) of 2 mg/mL $NaHCO_3$ solution of various initial pH values in 260 mL flasks.
The measurements were made from two flasks of each initial pH and amount of bicarbonate solution one day after addition of solution at 37°C. Note that for initial pH values up to 7.5, the increment remains constant on a particular level for each particular amount of solution.

	Thermo Scientific Nunc Lab uses				△ pH	
Thermo Scientific Nunc vessel	Culture area cm^2	Medium volume M mL	Air volume A mL	Total volume (A+M) mL	Measured ±0.1	Calculated
Tube 100 x 13 mm (roller)	32	7	2.5	9.5	0.6	0.33
Tube 100 x 14 mm (roller)	34	7	2.5	9.5	0.6	0.33
Tube 100 x 16 mm	6	3	8.5	11.5	0.9	0.92
SlideFlask	9	3	17	20	1.1	1.06
Flask 40 mL	25	7	33	40	1.1	1.03
Flask 50 mL	25	7	55	62	1.1	1.11
Flask 260 mL	80	30	235	265	1.1	1.11
Flask 800 mL	175	68	750	818	1.2	1.15
Singletray	630	200	700	900	1.0	0.97

Table 3
Measured and calculated pH increments, s pH, in MEM with Earle's salts of initial pH 7.0 in closed Thermo Scientific Nunc tissue culture vessels using Thermo Scientific Nunc Lab volumes of medium. The measurements were made one day after addition of medium at 37°C. With serum in the medium, slightly smaller increments would be measured. See text for further explanation.

vessel with CO_2 (through a sterile filter) before closing. To avoid the risk of overdosing CO_2 and making the medium too acidic, one may use prefabricated air mixtures containing the appropriate percentage of CO_2 instead of pure CO_2.

It should be noted that while the CO_2 level can easily be restored in media with a low buffering capacity, this type of medium is susceptible to rapid acidification by respiration and requires more frequent replacement than strongly buffered media. This disadvantage can sometimes be overcome by using bicarbonate in combination with the CO_2 independent organic buffer, HEPES.

In conclusion, CO_2 gassing becomes more important, the larger the A/(A + M) ratio (Table 3), the larger the bicarbonate concentration, the smaller the number of cells seeded, and the slower the respiration rate of the cells. If gassing is omitted, one should adjust the initial pH to the lowest possible value, taking into account the calculated pH increment and the optimal pH for the cell type in question.

Pressure

When a culture vessel is seeded with cells and medium at room temperature and then closed and incubated at 37°C, excess pressure will arise inside the vessel mainly due to heating of the confined air.

If preheated medium is used, the excess pressure will be due to air heating alone, whereas with medium at lower temperatures there will be an additional contribution from the pressure increase of saturated water vapor, which is dependant on the temperature and volume of the medium. Table 4 shows the total pressure increments with media of different temperatures.

On the other hand, there should be no contribution from cellular respiration as cells are normally fed with pure hydrocarbon (D-glucose) as an energy source, whose respiratory degradation consumes

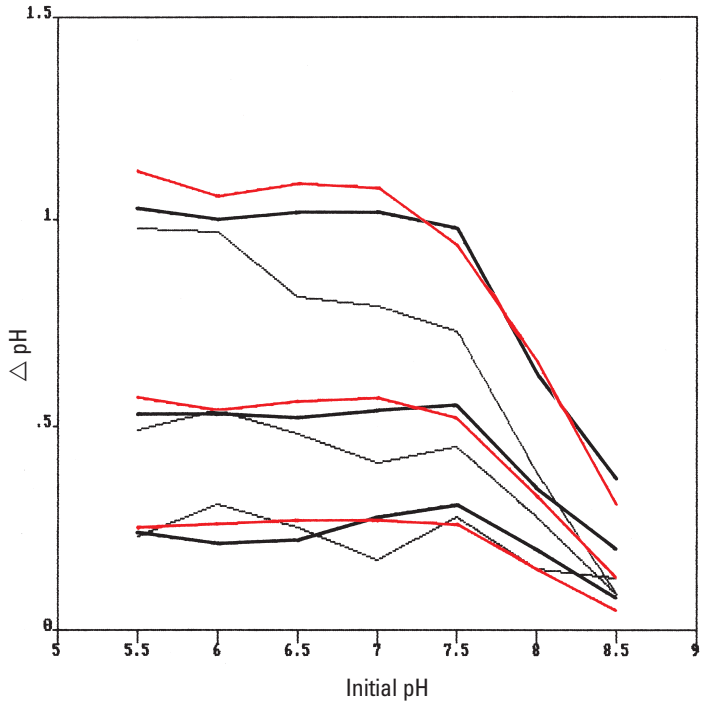

Fig. 4
Measured pH increments, △ pH, in 30 mL (upper three curves), 130 mL (middle three curves), and 230 mL (lower three curves) of 0.35 mg/mL $NaHCO_3$ (thin curves), 2.0 mg/mL $NaHCO_3$ (red curves), and 3.7 mg/mL $NaHCO_3$ (fat curves) of various initial pH in 260 mL flasks. The measurements were made one day after addition of $NaHCO_3$ solution at 37°C. Note that except for 30 mL of 0.35 mg/mL $NaHCO_3$ (upper thin curve), the increments seem to be independent of the bicarbonate concentration.

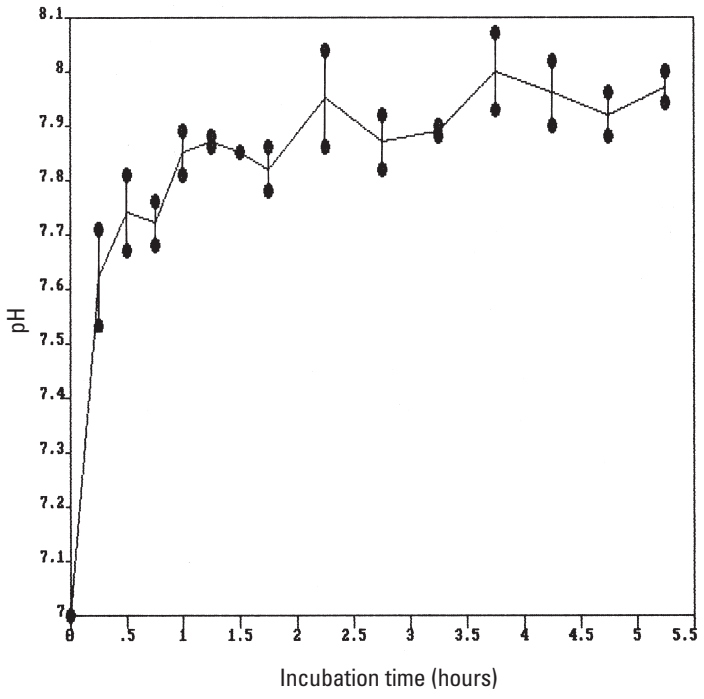

Fig. 5
Rising of pH in 30 mL MEM with Earle's salts and 10% foetal calf serum of initial pH 7.0 in 260 mL flask. The measurements were made from two flasks at a time after addition of medium to a series of flasks at 37°C. Note that the greater part of the pH rise occurs during the first hour after addition of medium.

O_2 and produces CO_2 in equimolar amounts:

$$C_6H_{12}O_6 + 6O_2 \xrightarrow{resp.} 6CO_2 + 6H_2O$$

Excess pressure in plastic culture vessels is undesirable as it may cause the vessels to crack. Large vessels are more sensitive in this respect because they are less rigid than smaller vessels. Especially, the 800 mL flasks may become so "pot-bellied" that they can pivot on their thickest point.

There are several ways to prevent excess pressure:

1. By carrying out all seeding operations with pre-warmed ingredients in a hot room. However, this assumes the presence of an entire hot room and implies very unpleasant working conditions.
2. By using pre-warmed medium and pre-warmed gassing mixture. However, the latter may not be feasible.
3. By ventilating the vessels momentarily when temperature equilibrium has been reached. However, this does not actually prevent excess pressure, only limits it to minimum duration without eliminating the risk of cracking. The risk could be reduced by frequent ventilation during temperature equalization, but this would be rather tedious.
4. By incubating the vessels with loosened closures until temperature equilibrium has been reached. This would be the easiest way to solve the problem, implying, however, a risk of contamination and escape of CO_2 (present from gassing).

Therefore, the 800 mL and 260 mL flasks have been equipped with closures that can be put in a well-defined ventilation position. In this position, the opening is sufficiently large to permit free equalization of pressure, but at the same time sufficiently narrow to ensure negligible contamination risk and minimal decrease of internal CO_2 percentage during the relatively brief temperature equilibration period of ½-1 hour.

For CO_2 gassing and pressure equalization of Thermo Scientific Nunc Singletray and Multitrays (Cell Factories), the inlets should be fitted with sterile air filters which may afterwards be closed by clamping tubing mounted on their outer connecting pieces.

More generally, gassed or ungassed vessels could be incubated with loosened closures in a CO_2 incubator for a period sufficiently long to permit temperature equilibration and establishment of the appropriate CO_2 percentage. However, this assumes the presence of a more sophisticated and expensive incubator with a CO_2 control.

Finally, it should be mentioned that complete temperature equilibration using loose fitting lids is necessary for Thermo Scientific Nunc MicroWell culture plates to be incubated sealed with sterile adhesive tape, because this sealing would not withstand excess pressure.

Initital medium temperature °C	Total pressure increment atm.
37	0.06
20	0.10
4	0.12

Table 4
Calculated total pressure increments in closed vessels with media of different initial temperatures when heated to 37°C, assuming that the air inside the vessel is initially 20°C. See text for further explanation.

Austria
+43 1 801 40 0
Belgium
+32 53 73 42 41
China
+86 21 68654588
Denmark
+45 4631 2000
France
+33 2 2803 2180
Germany
+49 6184 90 6940
India
+91 22 6716 2200
Italy
+39 02 02 95059 or 434-254-375
Japan
+81 3 3816 3355
Netherlands
+31 76 571 4440
Nordic/Baltic countries
+358 9 329 100
North America
+1 585-586-8800
Russia/CIS
+7 (812) 703 42 15
Spain/Portugal
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South America
+1 585 899 7298
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