Studies on the Ventilation in Submerged Fermentations

Part III. Behavior of Carbon Dioxide in the Fermentation and Dynamic Characteristics of Respiration in Submerged Culture

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By using the modified Severinghaus CO₂ electrode, the relationship between partial pressure of carbon dioxide in effluent gas and that in culturing system was investigated. Partial pressure of carbon dioxide in gas phase was almost equivalent to the average value of dissolved carbon dioxide tension in liquid phase for a given short time of the fermentation. The term of r_e was introduced in order to study the dynamic characteristics of carbon dioxide evolution in submerged fermentors. The dynamic characteristics of respiration in submerged fermentation was also studied by using biological r_{ab} and r_e .

In order to study the behavior of carbon dioxide to cultured system, dissolved carbon dioxide and bicarbonate ions in fermented broth were analyzed by using the modified instrument and apparatus. According to the experimental results described in the previous papers,^{1,2)} the formula of carbon dioxide in liquid phase could be classified into two groups, the gaseous form and the dissociated form. The quantity of gaseous carbon dioxide is expressed by Henry's law and it is in proportion to the partial pressure of carbon dioxide of bubbles in the fermentor.

By using Bunsen's absorption coefficient for carbon dioxide of the employed sample and the partial pressure of carbon dioxide in bubbles in the fermentor, the quantity of dissolved carbon dioxide ought therefore to be calculated. At the same time, the quantity of bicarbonate ion is indicated in Henderson-Hasselbalch's equation which is expressed in the function of the logarithm of the partial pressure of carbon dioxide in liquid phase.

Thus, the both gaseous carbon dioxide and bicarbonate ion were expressed as a function of carbon dioxide tension of bubbles in the fermentor.

Carbon dioxide tension in cultured system was directly detected by the Severinghaus CO₂ electrode which was commercially obtained from Beckman Instrument Co. The output of the electrode was amplified by the Beckman's physiological gas analyzer Model 160. The Severinghaus CO₂ electrode was installed to jar fermentors as well as the oxygen sensor,³⁾ and dissolved carbon dioxide tension and dissolved oxygen tension were measured independently.

Coexisting bicarbonate ions and other form of carbonate did not disturb the output of this instrument.

According to the information obtained by the experiment in this paper, the carbon dioxide tension in liquid phase in fermentors was equilibrated with the concentration of carbon dioxide in effluent gas at the same partial pressure. This fact was also confirmed

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2) A. Ishizaki, Y. Hirose and T. Shiro, *ibid.* **35**, 1852 (1971)

3) Y. Hirose, J. Nakamura, H. Okada and K. Kinoshita, *Agr. Biol. Chem.*, **29**, 931 (1965).

by the results of the determination of bicarbonate ion concentration in the cultured broth, because the determined bicarbonate ion concentration approximately agreed with the results of the calculation using the carbon dioxide concentration in effluent gas.

The behavior of carbon dioxide to submerged fermentations as well as that of bicarbonate ion was studied by the analysis of carbon dioxide of the effluent gas.

The method of the estimation of the respiration quotient in submerged fermentations was also established by the study of the calculation by using the data of oxygen concentration and carbon dioxide concentration of exhaust gas of fermentors.

MATERIALS AND METHODS

Microorganisms employed in this study were *Bacillus subtilis* sp.⁴⁾ and *Brevibacterium lactofermentum*.⁵⁾ Cultivations of these microorganisms were performed in the media described in the previous papers. Fermentation was carried out by the apparatus of Kobayashi *et al.* with a pH controlling means using gaseous ammonia.⁶⁾

Oxygen tension in liquid phase and effluent gas was measured by the oxygen analyzer, Beckman's Model 777, and oxygen absorption rate in the cultured system was estimated by the unsteady state gas analysis method reported by Hirose *et al.*^{7,8)}

Carbon dioxide tension in liquid phase was determined by the instrumentation, the details of which were described in the previous paper.

The concentration of carbon dioxide in effluent gas was detected by the infra-red gas analyzer whose principle was reported by Collier *et al.*⁹⁾

The amount of bicarbonate ion in cultured liquid was analyzed by the Warburg direct method which was developed previously.

RESULTS

1) Relationship between partial pressure of carbon dioxide in gas phase and that in liquid phase

The relationship between partial pressure of carbon dioxide in gas phase ($p\text{CO}_2$) and carbon dioxide tension in liquid phase (DCO_2) was investigated by employing inosine fermentation. The modified Severinghaus CO_2 electrode was installed in the jar fermentor and the signals from the electrode were amplified by the high impedance circuit. The behavior of carbon dioxide in liquid phase was accurately detected by this instrumentation.

A suitable amount of gaseous ammonia was fed to the fermentor so that the fermentation was carried out at the constant pH level except the beginning part and the ending part of the fermentation time and at those parts of the fermentation, the pH of the culturing liquid was increasing, provided the pH controller was operated with alkaline material feeding.

The DCO_2 tension of the culturing system was varied depending upon the pH change at all phases of the fermentation. It was said that this variation was made by the interconversion of bicarbonate and carbon dioxide whose quantity was given by Henderson-Hasselbalch's equation. These phenomena are demonstrated in Fig. 1 and Fig. 2 which are the recording-chart of pH and DCO_2 in pH controlled culturing system.

According to the experimental results, the partial pressure of carbon dioxide in effluent gas ($p\text{CO}_2$) was given by the average partial pressure of the maximum DCO_2 tension at the lowest pH value and the minimum DCO_2 tension at the highest pH value in one operation of neutralization in continuous pH control. It was therefore said that the partial pressure of carbon dioxide in gas phase was

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FIG. 1. Details of the Variation of Carbon Dioxide Tension in Liquid Phase Responded to pH Change (Inosine Fermentation).

FIG. 2. Recording Chart of pH and DCO_2 in Glutamic Acid Fermentation.

in the equilibrium state with the dissolved carbon dioxide tension at almost the same partial pressure.

The simultaneous trace of pCO_2 and DCO_2 in the reaction vessel for the interconversion is shown in Fig. 3. This chart shows that

FIG. 3. The Variation of Carbon Dioxide Tension in Liquid Phase and Gas Phase at the Interconversion of Bicarbonate and Carbon Dioxide.

DCO_2 tension is instantaneously increased to the maximum level but pCO_2 is gradually raised to reach the equilibrium state with the same partial pressure of carbon dioxide in liquid phase. In this manner, the partial pressure of carbon dioxide in effluent gas was in equilibrium at the same partial pressure of carbon dioxide in liquid phase.

This principle was applicable to the culturing system. In this connection, the partial pressure of carbon dioxide in effluent gas should also be in equilibrium with the average value of the maximum DCO_2 tension and the minimum DCO_2 tension in a given short period of fermentation time. This fact is shown in Fig. 4.

The relationship among pCO_2 , DCO_2 and pH with the fermentation time course is de-

monstrated in Fig. 5 employing inosine fermentation. The data show that the variation of DCO_2 tension is in response to pH change

of the cultured system, and the pCO_2 value of effluent gas is the same as the average DCO_2 tension in cultured liquid.

The reason for the fact that pCO_2 was given by the average value of the maximum DCO_2 and the minimum DCO_2 might be the large volume of the holding-up of gas in fermentors which had some disadvantages for the complete and instantaneous mixing and the distribution of evolved carbon dioxide from internals of the culturing system.

2) Bicarbonate concentration and DCO_2 tension in the cultured system

Bicarbonate concentration in glutamic acid fermentation broth and inosine fermentation broth was determined by the Warburg direct method. In the actual culturing system, the carbon dioxide concentration in effluent gas of fermentors was varied depending upon the rate of air flow.

The amount of bicarbonate ion in cultured broth was therefore given by the function of pCO_2 . In other words, bicarbonate concentration was in proportion to the partial pressure of carbon dioxide in effluent gas. The pH of broth was varied depending upon the physiological activity of microorganisms.

Thus, bicarbonate concentration in the cul-

FIG. 4. Recording Chart of pCO_2 and DCO_2 in Glutamic Acid Fermentation.

FIG. 5. The Relationship among pCO_2 , DCO_2 , pH and Fermentation Time Course in Inosine Fermentation.

Fig. 6. Behavior of Bicarbonate in Cultured System (Inosine Fermentation).

●—● 1/4 vvm ○—○ 1/2 vvm △—△ 1/1 vvm

tured system was influenced by $p\text{CO}_2$ of gas phase of fermentors and the pH of the cultured liquid.

The interconversion of bicarbonate ion and carbon dioxide always occurred if pH of liquid was varied, so that the feeding of gaseous ammonia for pH control was accompanying the DCO_2 change and the variation of bicarbonate ion concentration.

Figure 6 shows the influence of aeration on the bicarbonate ion concentration in inosine fermentation. The fermentation was carried out under the aeration conditions of 1/1 vvm, 1/2 vvm and 1/4 vvm at atmospheric pressure with the agitation of 1500 rpm for 300 ml of liquid. The $p\text{CO}_2$ in effluent gas was in inverse proportion to the volume of aeration, and the average concentration of bicarbonate ion was in proportion to the carbon dioxide partial pressure in exhaust gas.

Glutamic acid fermentation was performed by controlling pH at 8.0 and the concentration of bicarbonate ion in this fermentation was more than ten fold of that in inosine fermentation. The results are shown in Fig. 7. According to the data, the maximum bicarbonate concentration of glutamic acid fermentation was approximately 80 mM/liter at the aeration rate 1/4 vvm and almost a half of the

concentration at 1/2 vvm.

In the previous paper, it was reported that the bicarbonate concentration was substantially expressed by Henderson-Hasselbalch's equation in the culturing system, but the constants in the equation for the dissolution and dissociation of carbon dioxide in cultured system were found to be greatly different from the model liquid system such as phosphate buffer solution, glucose solution and fermentation medium.

The concentration of bicarbonate ion in the broth of inosine fermentation and glutamic acid fermentation was estimated by the modified equations by using the experimental pK' which was calculated by the relationship shown in Fig. 8.

The empirical equation was introduced by the solution for the function given by the relationship in Fig. 8, and two examples for both inosine fermentation and glutamic acid fermentation were indicated as follows:

For inosine fermentation finished broth,

$$\log \cdot [\text{HCO}_3^-] = \log (\text{CO}_2) + \text{pH} - 10.519 \quad (1)$$

or

$$[\text{HCO}_3^-] = \text{CO}_2 \text{ antilog } (\text{pH} - 10.519) \quad (1')$$

and for glutamic acid fermentation finished broth

FIG. 7. Behavior of Bicarbonate in Cultured System (L-Glutamic Acid Fermentation).

○—○ 1/4 vvm ●—● 1/2 vvm

$$\log \cdot [\text{HCO}_3^-] = \log (\text{CO}_2) + \text{pH} - 11.040 \quad (2)$$

or

$$[\text{HCO}_3^-] = \text{CO}_2 \text{ antilog } (\text{pH} - 11.040) \quad (2')$$

In the above equations, CO_2 is indicated in the molarity of dissolved carbon dioxide and $\text{p}K'$ is equal to $\text{p}K_{\text{HCO}_3^-} + 4.231 - \log \alpha$.

Owing to the above expressions, those empirical equations were considered to be the other forms of Henderson-Hasselbalch's law. The estimated concentration of bicarbonate ion in both inosine fermentation and glutamic acid fermentation were calculated by the above

equations. Those results are described in Table I. At the same time, the data obtained by the manometry are shown in the same table. Data show that the experimental results agree well with the theoretical values.

It was therefore said that the concentration of bicarbonate ion in cultured system varied depending upon pH and the quantity of dissolved carbon dioxide which was in proportion to the partial pressure of carbon dioxide in gas phase of fermentors.

In addition to this, Bunsen's absorption coefficient for carbon dioxide α and the index

TABLE I. DETERMINED BICARBONATE AND CALCULATED BICARBONATE IN THE CULTURED SYSTEM

Kinds of fermentation (Aeration rate)	hr	pH	pCO_2 atm	$\text{p}K_{\text{HCO}_3^-}$	α ml/ml	Calculated [HCO_3^-] mmole/ liter	Determined [HCO_3^-] mmole/ liter
Inosine (1/2 vvm)	3	6.48	0.005	6.185	0.615	0.27	0.42
	9	6.93	0.020	6.149	0.600	3.24	3.63
	23	6.52	0.030	6.109	0.590	2.04	2.19
	35	6.56	0.032	6.051	0.580	2.67	2.88
	48	7.35	0.017	6.051	0.580	8.75	9.10
	60	6.70		6.051	0.580		2.03
Glutamic acid (1/4 vvm)	6	7.80	0.015	6.460	0.670	10.0	10.0
	12	7.80	0.128	6.460	0.670	88.0	90.5
	18	7.80	0.120	6.539	0.670	68.3	68.5
	24	7.80	0.112	6.539	0.670	63.7	62.0
	36	7.80	0.045	6.635	0.670	20.5	20.0

Fig. 8. The Relationship between $p\text{CO}_2$ and HCO_3^- in Cultured System.

of the first stage dissociation constant for carbon dioxide $pK_{\text{HCO}_3^-}$ also varied as the fermentation went on, and bicarbonate concentration was affected by this variation.

It was concluded from the experimental results described above that the concentration of bicarbonate ion in cultured liquid was always maintained at the maximum level calculated by an empirical equation for the specified fermentation time with a given pH and $p\text{CO}_2$.

3) Method for the analysis of the dynamic characteristics of respiration in submerged fermentations

As described in the preceding section, the dissolution and dissociation of carbon dioxide in liquid phase of fermentors were always in the state of equilibrium, and the amount of evolved carbon dioxide from submerged fermentors was considered to be the product of respiration of living cells.

If a pH of broth was maintained at a constant level, both the quantity of evolved carbon dioxide and the concentration of bicarbonate ion in cultured liquid were given as the function of the partial pressure of carbon dioxide in effluent gas.

The quantitative studies of oxygen absorption rate and carbon dioxide elimination rate were the fundamental subjects for the analysis of the dynamic characteristics of respiration in submerged fermentations. Behavior of oxygen to submerged fermentations was investigated by Hirose *et al.* by using the dissolved oxygen analyzer.

According to the fact obtained by the authors, the behavior of carbon dioxide including bicarbonate ion in submerged fermentors was possible to study by using the data of the determination of the partial pressure of carbon dioxide of effluent gas. The dynamic characteristics of respiration in submerged fermentation was also possible to analyze by using the data of exhaust gas analysis.

The concentration of bicarbonate ion in culturing liquid varied as the fermentation went on, but this variation did not affect the balance of the mass transfer of carbon dioxide in the culturing system provided difference in the concentration of bicarbonate ion between the starting period of the fermentation and the final period of the fermentation was not extremely large. The function of the partial pressure of carbon dioxide in effluent gas was differentiated to give the dynamic characteristics of carbon dioxide evolution in submerged fermentations.

The amount of eliminated carbon dioxide from broth into effluent gas in one minute was evaluated by the following equation,

$$(CO_2) = \frac{(pCO_2)_0}{P_0} Q_0 - \frac{(pCO_2)_1}{P_1} Q_1 \text{ ml/min} \quad (3)$$

where $(pCO_2)_0$ is the partial pressure of carbon dioxide in exhaust gas, P_0 is the total pressure of exhaust gas, Q_0 is the quantity of exhaust gas at the standard state, $(pCO_2)_1$ is the partial pressure of carbon dioxide in introduced air, P_1 is the total pressure of introduced air and Q_1 is the volume of aeration at the standard state.

The concentration of carbon dioxide in atmospheric air is very small so that the term of $(pCO_2)_1$ is considered to be zero as compared with $(pCO_2)_0$. As far as the respiratory quotient of the fermentation holds at approximately one, Q_0 is regarded as same as Q_1 . The equation (3) is thus approximated by the equation stated below,

$$(CO_2) = \frac{pCO_2}{P} \cdot Q_1 \text{ ml/min} \quad (4)$$

where pCO_2 is the partial pressure of carbon dioxide in gas phase of the fermentor and P is the total pressure of the culturing system.

The amount of carbon dioxide evolution from a unit volume of the culturing liquid is then given by the equation,

$$\begin{aligned} (CO_2) &= \frac{Q_1}{V_1} \frac{pCO_2}{P} \\ &= N \frac{pCO_2}{P} \text{ ml/ml min} \end{aligned} \quad (5)$$

where V_1 is the volume of liquid in the fermentor and N is the index of the aeration rate (vvm).

The molarity of carbon dioxide evolution in a unit volume and in a unit time is therefore expressed in the following equation.

$$(CO_2) = \frac{1}{22.4} N \frac{pCO_2}{P} 10^{-3} \text{ mole/ml} \cdot \text{min} \quad (6)$$

The total carbonate in liquid phase is given by the sum of dissolved carbon dioxide and bicarbonate ion so that the whole mass transfer of carbon dioxide in submerged fermentations is expressed in the following equations in which the quantities of the interconversion of bicarbonate ion and carbon dioxide are taken into account.

At first, the quantity of the interconversion due to the change of the partial pressure of carbon dioxide is given by the equation as follows.²¹

$$\begin{aligned} &\frac{d(CO_2) \text{ converted}}{dt} \\ &= \frac{\alpha}{22.4} \left\{ 1 + \text{antilog} \left(pH + \frac{A\sqrt{I}}{1+B \cdot a \cdot \sqrt{I}} \right. \right. \\ &\quad \left. \left. - pK' \right) \right\} \frac{dpCO_2}{dt} 10^{-3} \text{ mole/ml} \cdot \text{min} \quad (7) \end{aligned}$$

The sum of equations (6) and (7) consequently give the absolute quantity of evolved carbon dioxide in a unit time.

$$\begin{aligned} (CO_2) \text{ evolved} &= \frac{1}{22.4} N \frac{pCO_2}{P} 10^{-3} \\ &+ \frac{\alpha}{22.4} \left\{ 1 + \text{antilog} \left(pH + \frac{A\sqrt{I}}{1+B \cdot a \cdot \sqrt{I}} - pK' \right) \right\} \\ &\times \frac{dpCO_2}{dt} 10^{-3} \text{ mole/ml} \cdot \text{min} \end{aligned} \quad (8)$$

In the steady state of fermentation, the concentration of carbon dioxide in exhaust gas was maintained at almost the same level. In this case, the term of $dpCO_2/dt$ can be regarded as zero and the above calculation is approximated by the following equation.

$$(CO_2) \text{ evolved} \doteq \frac{1}{22.4} N \frac{pCO_2}{P} 10^{-3} \text{ mole/ml} \cdot \text{min} \quad (9)$$

It is said that this equation is the same as the formula for the calculation of the oxygen absorption rate in submerged fermentation proposed by Hirose *et al.*, in its dimension.

The new term of r_e is introduced for the expression of carbon dioxide evolution in submerged fermentations.

FIG. 9. r_{ab} and r_e in Inosine Fermentation (Aeration 1/2 vvm).

$$r_e = \frac{1}{22.4} \frac{Q_i}{V_1} \frac{pCO_2}{P} \cdot 10^{-3} \quad \text{mole/ml} \cdot \text{min} \quad (10)$$

The dynamic characteristics of the respiration of microorganisms in submerged fermentations $(RQ)_t$ is therefore expressed by the following equation,

$$(RQ)_t = \frac{r_e}{r_{ab}} = \frac{pCO_2}{P_1 - P_B} \quad (11)$$

where r_{ab} is the biological oxygen absorption rate, P_1 is the partial pressure of oxygen of the inlet of air and P_B is the partial pressure of oxygen of the outlet of air of fermentor. The variation of the quantity of dissolved oxygen and carbon dioxide is negligibly small as compared with that of r_{ab} and r_e . Thus, if a pH of the culturing system is maintained at a constant level, the respiratory quotient in submerged fermentation can be estimated by the value of $(RQ)_t$.

An example of these relationship in inosine fermentation is presented in Fig. 9.

DISCUSSIONS

The variation of DCO_2 tension in fermentors was precisely detected by employing the improved Severinghaus CO_2 electrode. At the same time, the partial pressure of carbon di-

oxide in exhaust gas was measured by the infra-red gas analyzer and the concentration of bicarbonate ion in cultured liquid was analyzed by the Warburg direct method. The behavior of carbon dioxide in submerged fermentation was thoroughly studied by the analysis of the above results.

On the other hand, the oxygen concentration in gas phase and in liquid phase was analyzed by Beckman's oxygen analyzer and oxygen absorption rate in the biological system was estimated by the unsteady state method.

According to the results of the determination of carbon dioxide in cultured system, the equilibrium of carbon dioxide was established on almost the same partial pressure between dissolved carbon dioxide tension in liquid phase and carbon dioxide concentration in effluent gas. This fact was proved by the experimental results on the manometry for the bicarbonate determination in both the inosine fermentation broth and the glutamic acid fermentation broth. In this view point, the behavior of gaseous carbon dioxide to liquid phase of submerged fermentations seemed to be quite different from that of oxygen to liquid phase of cultured system.

This phenomena might be caused by the reason that the velocity constant for the reaction of $CO_2 \longrightarrow HCO_3^-$ was greater than

that for the reversed reaction, $\text{HCO}_3^- \longrightarrow \text{CO}_2$.

The reaction steps of the dissolution and dissociation of carbon dioxide are different from the dissolution of oxygen. Taking into account the above matters, the scheme of the liberation of carbon dioxide is proposed as follows:

This mechanism is illustrated in Fig. 10.

The analytical results showed that the concentration of bicarbonate ion in cultured system was maintained at the highest value derived from the calculation using the empirical equations. This fact can be said to be one of the evidence to support the above assumption.

It was found that the concentration of bicarbonate ion in liquid phase of submerged fermentation could be estimated by the computation substituting pH and pCO_2 of the fermentation for the empirical Henderson-Hasselbalch's equation.

FIG. 10. Mechanism of CO_2 Elimination in Submerged Fermentor.

The method for the studies of the dynamic characteristics of respiration in submerged fermentation was also established by using the fact obtained in the experiment.

The new term of r_e was introduced for the expression of the dynamic characteristics of the carbon dioxide evolution. The respiratory quotient of submerged fermentations was estimated by the function of biological r_{ab} and r_e .

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