STUDIES ON THE METABOLISM OF THE MAMALIAN OVA I. METHOD OF APPLICATION BY CARTESIAN DIVER

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I. METHOD OF APPLICATION BY CARTESIAN DIVER

By

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Introduction

In 1937, the ultra-micromanometry, i.g. Cartesian diver manometry by which the gas change of the order of the magnitude about $10^{-3}\mu l$ be measured was first published by Linderstrøm-Lang (I). Thereafter, this manometry was improved by Linderstrøm-Lang and Glick (2), Linderstrøm-Lang (3) Holter (4), Zeuthen (5, 6) and Anfisen (7), and applied to the measurement of the respiratory activity of a single cell.

The application of the diver method to the biological problem was performed by Boell, Needham and Rogers (8) and Boell, Kock and Needham (9). Nowadays, this manometer is required as the instrument necessary for the investigations in the field of microbiological problems, such as mammalian ova and α-cell or β-cell of Langelhans island etc.

Little is known of the metabolism of mammalian ova, except for the reports of Smith and Kleiber (10) and Fridhandler *et al.* (11–13) in the rabbit, and Boell and Nicholas (14) in the rat.

In the present experiments, we deal with the applicability or suitability of the Cartesian diver method which was modified by the authors to facilitate measurement of the respiratory activity of the mammalian ova. The applicability and the validity of the Cartesian diver modified are discussed in comparison with the Warburg manometry.

Materials and Methods

Oxygen consumption: Cartesian diver manomter used in the present study is shown in Fig. 1. The operation of this manometer is carried out essentially by Holter's method. According to Linderstrøm-Lang, it is desirable

that all experiments with divers are carried out in a room where the temperature is kept 2°C below the thermostat and the temperature of thermostat is regulated with a precision of 0.01°C. The original method reported by Linderstrøm-Lang and Holter is carried out in a thermostat kept at 22.5°C, because the object of the experiment is aquatic animals, such as amoeba, sea-urchin and amphibian eggs. If man performs the work with the diver method in the study of mammals under the conditions recommended by Linderstrøm-Lang, the temperature of the thermostat must be kept at 37.5°C and the room temperature where the experiment is carried out may be kept at 35.5°C, because the object of the experiment is the mammalian ova. But, in practice, it is impossible for man to endure the experimental periods in the room where the temperature is kept at 35.5°C. In the present study, therefore, all experiments with diver were carried out in a room where the temperature is kept at 23°C or is defined below 23°C.

The more the difference of temperature between the thermostat and room where the operations of the diver carried out, the larger the changes of the gas volume constant of the diver may be occurs. When the divers which were set in a room the temperature is kept at 23.0°C is introduced into the their floatation vessles (37.5°C), expansion of the gas phase in the diver occurs and the position of the charges (neck seals and mouth seal) in the divers rises. When the constant of the diver varied, the initial equilibrium pressure of the diver is very difficult to obtain because its buoyancy increases by the expansion of the gas phase.

Therefore, when the experiment is carried out under such a condition as the temperature difference between the room where the diver is set and thermostat in which gas volume change is measured is large, the solutions for sealing the neck and mouth must be filled to the level where the lower meniscus calculated from the formula will locate, because the level of the lower meniscus will be moved in proportion to the temperature difference between the room and thermostat and the constant of the diver must be calibrated at the temperature of measurement.

We attempted to determine the transferred rate of the position of the neck seals and mouth seel by the following formula, so that setting of the neck seal in the diver can be made easily. Where ΔV expresses the expansion rate of gas volume in the diver.

$$\Delta V = \frac{V \times T}{273 + T^0}$$

ΔV = the expansion rate of gas volume in the diver.
V = the gas volume of lowest neck seal meniscus.
T = difference of temperature between the thermostat and room.

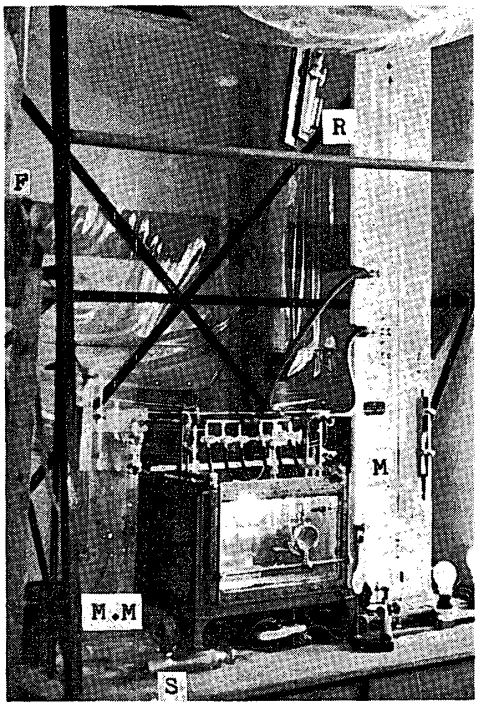
T^0 = temperature of the thermostat.

Therefore, the transfersed rate (ΔH) of the neck seal is calculated by the following formula.

$$\Delta H(\text{mm}) = \frac{\Delta V}{A}$$

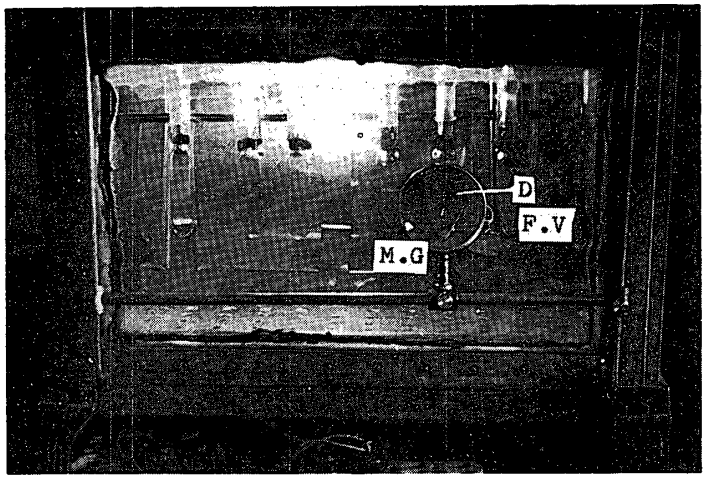
where A = cross sectional area (mm^2) of column of diver neck.

Because all divers used in the present study were the standard diver (flask form), the transferred rate of the neck seals and constant of the diver volume were calculated as follows. First, the total volume of the diver and



A

M : Manometer.
M.M : Mercury manometer.
S : 100 *ml.* Syringe.
(Pressure regulator)
R : Room regulator.
F : Fan.
M.G : Magnifying glass.
F.V : Floatation vessel.
D : Diver.



B

Fig. 1. Apparatus of Cartesian diver.

the volume of the diver bulb were determined by the weighing methods. In the operation with the diver, when the neck seals are charged by the filling pipette, the lower meniscus of the neck seals (e.g. NaOH as the carbon dioxide absorption reagent) is placed just on the line of the diver bulb and diver neck, but it frequently happens that the lower meniscus of the neck seals is not charged just on the border line of the diver bulb and diver necks.

In these cases, the length from the borderline of the diver bulb to the lower meniscus of the neck seal were measuresd by the measuring microscope, the gas volume of these parts were calculated. The changed rate of the neck seal and diver constant were determined according to the formula mentioned previously.

The true constant of the diver was determined by subtracting the volume of all the seal solutions charged from the total volume of the diver and then correcting the gas volume calculated from the formula and thus the basometric pressure and room temperature at this time to the pressure P and temperature t°C were found.

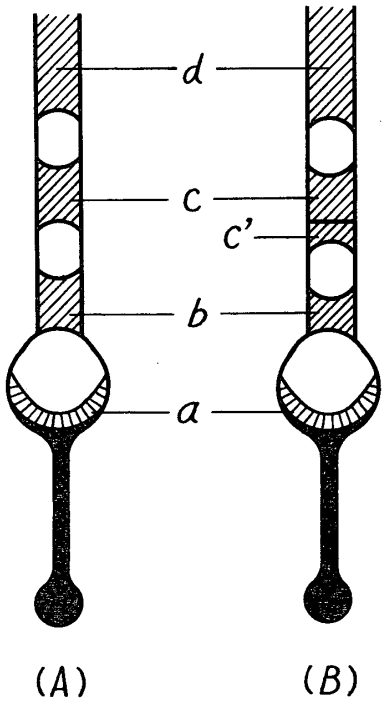


Fig. 2. Schematical drawing of arrangement of the divers used in the present study.

(A) Diver used for the oxygen consumption.
a : bottom drop.
b : N/10 NaOH.
c : Liquid paraffin.
d : mouth seal.

(B) Diver used for the Carbon dioxide evolution test.
a : bottom drop.
b : 2N HCl.
c′ : Piston acid (2N HCl)
c : Liquid paraffin.
d : mouth seal.

Measurements of oxygen consumption of E. coli : A samples of E. coli was washed several times by the Krebs-Ringer-Phosphate Buffer (pH 7.4) after 24 hrs. incubation and starvated during 24 hours in an electric refrigerator. E. coli suspension was prepared as a suspension containing 13 mg of bacteria of wet weight per *ml.* and 2μl of the suspension was used for Catersian diver manometry and 2*ml* of the suspension for the Warburg manometry.

Test of carbon dioxide evolution: 224μl per *ml* of $NaHCO_3$ as the standard solution was prepared. And, 2μl of the standard solution was used in the case of diver method and 2*ml* of the standard solution for the Warburg manometry.

The set of divers is shown in Fig 2. 2N hydrochloric acid for the evolution of carbon dioxide was filled as the lower neck seal.

The experiments were made of regular duration, and readings of equilibrium pressure of the diver at regular intervals.

Mixing procedure of acid: the apparatus employed for the mixing the reaction solution is shown in Fig 1. (A, B).

Mixing procedure used was the method reported by Anfisen. (7) At 60 minutes after incubation, the gradual application of pressure of 20 to 35 mm Hg. to Cartesian divers by 100 *ml* of the syringe were made. After the mixing of acid with the reaction solution, readings of equilibrium pressure of the diver were made at a minute intervals until the constant equilibrium pressure of the diver was obtained.

Sample for the Warburg manometry was 1000 fold that used for the oxygen consumption and carbon dioxide evolution in the case of diver manometry.

Results and Discussions

1) The equilibrium pressure of control diver.

The temperature of the thermostat is kept at 37.5°C and the heating lamp is a 60 watt chiken lamp controlled by the thermoregulator of L-form, "Shimazu", the precision of which is 0.01°C. The changes of readings of the equilibrium pressure in the control diver made at one minute interval are given in Fig. 3.

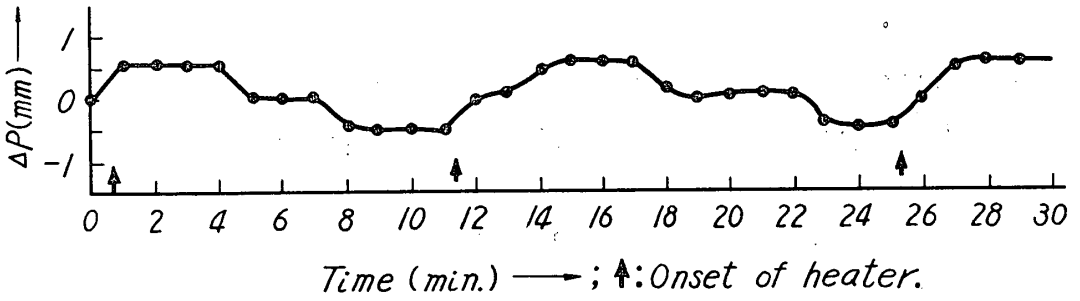


Fig. 3. Changes of the equilibrium pressure in the control diver.

As apparent from Fig. 3, the changes of the equilibrium pressure in the control diver were related to the onset or offsetting of the heating lamp. These changes of the equilibrium pressure in the control divers were in the order of ± 0.3 to 0.5 mm, and agreed with the results obtained by Linderstrøm-Lang *et al.* (2)

It appears to suggest that the regulation of the temperature in the thermostat was correct.

2) Oxygen consumption by the diver method.

a) Oxygen consumption of E. coli by each manometry.

As shown in Table 2, oxygen consumption of E. coli by the diver method agreed with the results obtained by the Warburg method. Therefore, it will be suggested that the measurement of the oxygen consumption by the diver

method under the present experimental conditions can be measured, and that the reproducing ability of the measurements is very good.

Table 1. Conditions of the measurement in each manometry.

	Diver method	Warburg method
Samples (bacterial suspension)	2 μl	2 ml
N/10 NaOH	0.9 μl	0.2 ml
Liquid paraffin	1.5 μl	–
Mouth seal	3.5 μl	–
Gas phase	air	air
Shaking	none	none

Table 2. Oxygen consumption of the starvated E. coli by the diver method and Warburg manometry.

Number of experiments	Manometer	
	Warburg method (μl/2 hr)	Diver method($\times 10^{-3}$ μl/2 hr)
1	287.68	285.92
2	281.34	247.03
3		266.50

b) Oxygen consumption of the mammalian ova.

(i) Oxygen consumption of the two-cell stage ova of the rabbit.

As mentioned above, it was established that the oxygen consumption of E. coli by the diver method can be measured, on the base of the comparison with the results of each method, i.e. the diver method and Warburg method.

The next experiment was carried out to determine the egg number required for the measurement of the oxygen consumption in the mammalian ova.

The rabbit ova were collected as follows: adult female rabbits were treated with P.M.S. (Anteron, Schering Co.) according to the method reported by Sakuma *et al.* (15), mated with the vigorous buck, slaughtered at 24 hours after the matings and the oviducts were removed. The contents of the oviducts flushed out into the Petri dish by Krebs-Ringer-Phosphate Buffer (pH=7.2) containing 0.1 per cent glucose. The ova were picked up individually from the Petri dish by means of a capillary pipette with a tuberculin syringe.

After the desired number for the measurement of the oxygen consumption were obtained, the ova were washed through several changes of Krebs-Ringer-Solution to remove the epithelial cells which flushed out with the ova from the oviduct. The ova with the medium were pipetted into the divers with a breaking pipette (4).

The control divers were charged with the flushed medium of the ova. Dimension of the divers used for the measurements of the oxygen consumption in the rabbit ova is shown in Table 3.

Table 3. Dimension of the diver used for the measurement of the oxygen consumption in the rabbit ova.

	Total Volume of diver (μl)	Seals (μl)				Constant of Diver (μl)	Changes of equilibrium pressure (mm)	$Q_0 2/28$ ova/hr	$\times 10^{-3}\,\mu l$/ ovum/hr
		Buffer + (ova)	N/10 NaOH	Oil	Mouth				
Control	22.15	1.0	0.9	1.1	4.0	13.82	-	-	-
Sample	21.60	1.2	0.9	1.0	4.2	12.40	22	27.28	0.97

Because of the samllness of the respiratory activity in the ova, the changes of the equilibrium pressure in the divers were made at 20 minutes interval between readings.

The changes of the equilibrium pressure in the sample diver (rabbit ova) and the control diver are shown in Fig. 4.

As apparent from Fig. 4, the equilibrium pressure of the control diver was not changed, but that of the sample diver increased linearly as the experimental period progressed.

It may be suggested that the respiratory activity of the ova progressed at a constant rate, and that the ova lived during the experimental period. From these results of the equilibrium pressure changes in the sample diver, it was cleared that the respiratory activity of the ova can be measured sufficiently with the use of about 28 ova of rabbit by the Cartesian diver manometry. It would be suggested that the oxygen consumption of the mammalian ova

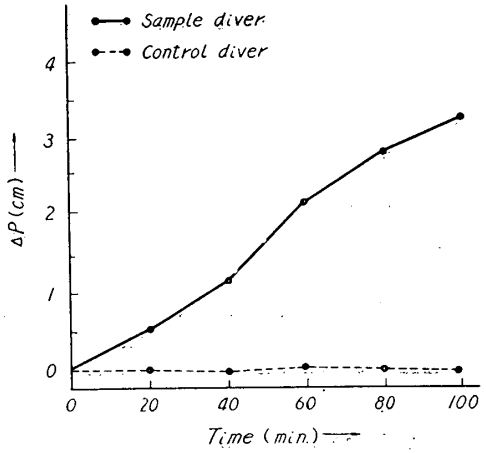


Fig. 4. Changes of the equilibrium pressure in the sample diver (rabbit ova) and control diver.

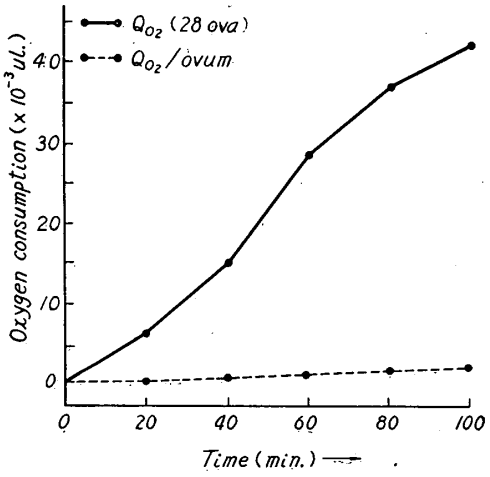


Fig. 5. Oxygen consumption of the rabbit ova at the two-cell stage.

can be measured easily with the several numbers (10 to 13) of ova, so long as the total volume of the diver is about one third of that used in the present study.

The oxygen consumption of the rabbit ova are given in Fig. 5. The oxygen consumption per rabbit ovum calculated from Fig. 5 was 0.97×10^{-3} μl/hour.

There is very little literature on the oxygen consumption of the rabbit ova: Smith and Kleiber (10) and Fridhandler, Hafez and Pincus (11-13).

The oxygen consumption of the rabbit ova obtained in the present experiment was higher than that reported by Fridhandler *et al.* ($0.61\times10^{-3}\mu l$/ovum/hr.), but the values were much lower than those given by Smith and Kleiber ($26.0\times10\mu l$/ovum/hr.). As has been discussed by Fridhandler *et al.* (11), the difference in each finding will probably be due to the different experimental conditions employed (incubating medium, gas phase and temperature etc).

(*ii*) *Oxygen consumption of the rat ova at the blastocyst stage.*

The rat ova at the blastocyst stage were collected from the uterus of the females sacrificed at 115 to 120 hours after the observation of the vaginal plug.

The operations of the diver method were carried out as well as in the rabbit ova. The dimensions of the diver used for the measurement of the oxygen consumption in the rat ova is shown in Table 4.

Table 4. Dimension of the diver used for the measurement of the oxygen consumption in the rat ova.

	Total volume of diver (μl)	Seals (μl)				Constant of diver (μl)	Changes of equilibrium pressure (mm)	$Q_0 2/4$ ova/hr	$\times10^{-3}\mu l$/ovum/hr
		Buffer + (ova)	N/10 NaOH	Oil	Mouth				
Control	22.15	0.8	1.0	1.5	4.0	13.10	-	-	-
Sample	21.60	0.85	1.1	1.8	3.8	12.98	6	7.788	1.947

The results of the oxygen consumption of the rat ova are given in Fig. 6 and Fig. 7. As apparent from Fig. 6, the equilibrium pressure in the sample diver changed linearly at the constant rate of 0.6 cm per hour.

Because the changes of the equilibrium pressure in the sample diver was 0.6 cm per hour, it means that the oxygen consumption of the rat ova (four ova) can be measured under the conditions shown in Table 4. But, the greater the difference of the equilibrium pressure between the sample and control divers is, the easier the measurement with the diver becomes, and the easier the correction becomes.

Therefore, it seems to be desirable that the dimension of the diver for the measurements of the oxygen consumption in the mammalian ova is as

small as posible.

From the results of this experiments, it will be said that the number of the ova for the measurement of the respiratory activity at the blastocyst stage is desirable to be of several numbers of them.

There is no literature on the oxygen consumption of the rat ova at the blastocyst stage, but the studies on the respiration of the blastocyst stage of the rabbit ova have been undertaken by Fridhandler *et al.* (11). According to their results, a sudden increase in the oxygen consumption at the blastocyst stage had occurred, suggesting that the oxygen consumption of the rat ova must be increased considerably at that stage, and that the oxygen consumption of that stage can be measured with several numbers of ova.

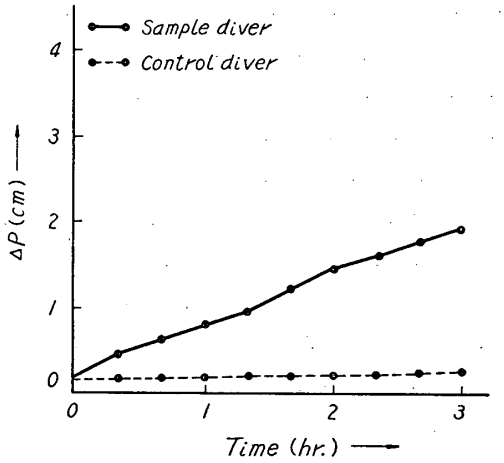


Fig. 6. Changes of the equilibrium pressure in the sample diver (rat ova 4) and control diver.

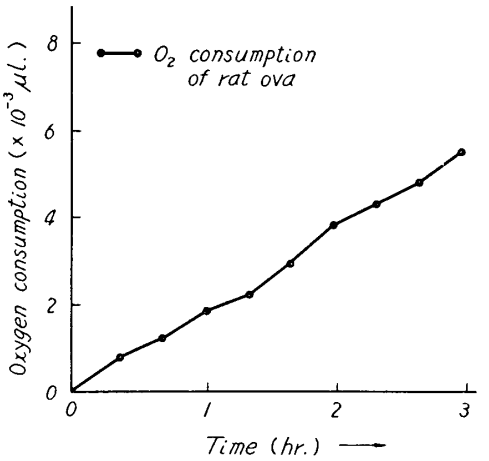


Fig. 7. Oxygen consumption of the rat ova at the blastocyst stage.

3) *Carbon dioxide evolution test by the diver method.*

On the carbon dioxide evolution test, the results obtained from the experiments in the diver method was as good as that of the Warburg method, that is, the recovery of the carbon dioxide in the each method was 95.2 per cent in the diver method and 95.0 per cent in the Warburg method.

Thus it was cleared that the carbon dioxide evolution test by the diver method under the conditions as described already is effective for the reproducing ability.

The detailed results on the carbon dioxide evolution test will be given in another paper.

Summary

In this investigation, the validity and applicability of the Cartesian diver method slightly modified by the present authors by means of the oxygen consumption with E. coli suspension and the carbon dioxide evolution test

with the standard of $NaHCO_3$ in comparison with Warburg manometry were carried out, and at the same time effort was made as to whether the method in problem can be applicable for the measurements of the respiratory activity in the mammalian ova.

The results are summarized as follows:

1. The results of oxygen consumption and carbon dioxide evolution test by the Cartesian diver method modified slightly by the present authors showed higher reproducing ability, and it was cleared that the method can be employed for the measurement of the metabolic activity in various biological problems.
2. The oxygen consumption of the rabbit ova at the two-cell stage (28 ova), obtained by the method mentioned above was 27.28×10^{-3} μl/hour, and the oxygen consumption/ovum/hour. was 0.97×10^{-3} μl.
3. The equilibrium pressure in the sample diver with the rat blastocyst (4 ova) increased at the rate of 0.6 cm per hour, and the oxygen consumption of the rat blastocyst, calculated from the base on the change of the equilibrium prsesure, was 7.788×10^{-3} μl/hour.

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