STUDIES ON THE METABOLISM OF THE MAMMALIAN OVA II. OXYGEN CONSUMPTION OF THE CLEAVED OVA OF THE RAT

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II. OXYGEN CONSUMPTION OF THE CLEAVED OVA OF THE RAT

By

Shichiro SUGAWARA and Motoyoshi UMEZU

Department of Animal Husbandry, Faculty of Agriculture, Tohoku University, Sendai Japan

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Introduction

Studies on the metabolic mechanism of the development of the living organims and on the biochemical process of embryology have been widely carried out on the sea-urchin, amphibia, fish and bird. Metabolic studies (respiratory activity) on the mammalian ova have been investigated by Boell and Nicholas (1), Fridhandler, Hafez and Pincus (2-4), and Smith and Kleiber (5).

Since the mammals are viviparous, the ova production is limited in small number and the ovulation is restricted only on a specific phase of the sexual cycle, a collection of ova sufficient to measure their respiration has been difficult. Therefore, the metabolic studies on them have been retarded.

However, a method to obtain many ova at one time regardless of phase of estrus or anestrus by the treatment of gonadotrophins; FSH and LH, was confirmed by Pincus (6) and Chang (7).

In addition, the development and improvement of the micromanometric techique (Cartesian diver method) has made possible the estimation of the respiration of a few cells. Accordingly, a study concerning the respiration of the mammalian ova became also possible.

Recently, the artificial pregnancy technique (transplantation of the fertilized ova), which have been first successfully performed by Heape (8) on the rabbit and subsequently by Pincus (9) and Chang (7) on the experimental animals, was investigated by Warwick and Berry (10), Hunter *et al.* (11, 12), Robinson and Adams (13), Averill (14) Kvasnikii (15) and Willett *et al.* (15, 16) on the farm animals, such as goat, sheep, swine and cow.

It seems possible that this technique is applicable for the improvement of the farm animals and their breeding. As reviewed by Hammond (17) and Willett (18), however, some of the problems for the sucessuful application of

the technique still remain unsolved. They are a) the storage of the fertilized ova, b) superovulation, c) the operation of the ova transfer and d) fertilization of the ova in vitro etc. Therefore, these problems must be solved to apply this technique as the artificial insemination in the field of the animal husbandry.

This series of investigation was planned to solve some of the problems mentioned above for the improvement of the artificial pregnancy technique and reproductive disorder in the field of animal husbandry.

In this paper, we have dealt with the changes in the respiratory activity of the rat ova during the early stage of the development.

Materials and Methods

Experimental animals: The oestrous cycles of the adult female rats of Wester-strain, weighing from 150 to 200 g, were accurately checked by the vaginal smear method every morning for about two weeks, and the rats which exhibited a regular oestrous cycle were used for the experiments.

Collection of the rat ova: When the many nucleated epithelial cells and some cornified cells found in the vaginal smear of the female at the prooestrus stage, she was placed with a vigorous male and allowed to mate overnight.

The rats, in which many sperms were found in the vaginal smear the next morning, were successively killed by decapitation at 24, 48, 72, 96, 120, 144 and 168 hours intervals after the observation of the vaginal plug formation.

For the collection of the ova ranging from the one-cell to the 16-cell stage, a capillary tube with a syringe was inserted into the cavity of the uterine with the oviduct, which was cut off at the sites of the Fallopian tube end and the cervix, and the contents of the oviducts were flushed out into a Petri dish with about 0.5 to 1 *ml* of $CaCl_2$-free Krebs-Ringer-Phosphate Buffer (pH=7.4) containing 0.1 percent glucose. The rat ova of the morula stage and blastula stage were flushed out from the uterine-tube junction and uterus, respectively.

Unfertilized ova were obtained from the rat mating with the vasectomized male at 11 to 24 hours after the observation of vaginal plug formation and the rat at 24 hours after the oestrous stage.

Ovulation numbers were plotted against the fresh corpora lutea in the ovary.

Measurement of oxygen consumption: The oxygen consumption was measured in the Cartesian diver apparatus (Holter (19)), which was slightly modified by the authors (20). The collected ova were washed with $CaCl_2$-free Krebs-Ringer-Phosphate buffer several times to remove the epithelial cells of the oviduct and uterus and were pipetted into the divers using a breaking pipette (19). The total volume of the diver ranged from 7 to 10 μl. The

volume of the "buffer+sample" in the diver ranged from 0.9 to 1.2 μl, and each diver contained 7-18 ova.

The air was used as a gas phase, since Bishop (21) reported that the oxygen tension in the reproductive tract (oviduct of the female rabbit) determined by the electrical method was in aerobic conditions (oxygen tension: 40 mm Hg.), also the present authors (22) made measurements of the oxygen tension in the uterine lumen of the rat by the Redox-indicator method (velocity of the oxidation from the leco-methylene blue to oxi-form) and micro-electrode, and confirmed that oxygen tension in the rat uterine lumen was 53 mmHg at the oestrous stage and aerobic conditions.

The temperature of the water bath was 37.5°C. All the operations of the diver were carried out in a room temperature kept at 23°C.

Results and Discussions

1. Collection of ova.

As the first step of this investigation, an effort was made as to whether the rat ova ranging from the one-cell to blastura stage for the measurement of the oxygen consumption could be easily obtained.

General aspects of the ova collected from the oviduct and uterus of the rat at 24, 48, 72, 96, 120, 144 and 168 hours intervals after the observation of copulation plug formation, are given in Table 1. The blood points in the ovary of the rat could not be easily distingushed as compared with those of the rabbit, guinea-pig, swine and other experimental animals. However, the fresh corpora lutea which had blood vessel at the time over 24 hours after the end of oestrous could be distigushed under the lupe from the old-corpora lutea which indicated the points of ovulation in the previous oestrous.

Therefore, the fresh corpora lutea in the ovaries of the rat, were regarded as the blood points, so that the ovulation number and the recovery percent of the ova were represented as the ratio of the collected ova against the fresh corpora lutea.

i) Recovery percent of the rat tubal ova.

As shown in Table 1, the recovery percent of the rat ova at the desired periods after the observation of mating plug were 88, 87, 82, 80, 84, 88, 8, 0, respectively. Therefore, the present method, in which the contents of the oviduct were flushed out from the side of uterus, may be said to be more suitable for the collection of the ova from the small animals, such as rat and mouse, as compared with the common method which had been used for the collection of the ova from the small experimental animals, and in which the oviduct was cut off and ova suspended.

The recovery percent of the ova was 88 at the highest. This is probably due to the error in the counts of the fresh corpora lutea in the rat ovary and

Table 1. General aspects of the ova collected.

Number of experiments	Hours after the observation of copulation plug	Site of recovery	Total number of corpus luteum	Total number of recovered ova	Cell stage of ova* (cleveage)	Percent of recovery	Mean
4	8	upper oviduct	34	30	1-cell...30	78-100	88.0
3	11	upper oviduct	31	27	1-cell...25 ab. 1	75- 90	86.9
9	24	oviduct	67	56	1-cell...13 2.....39 ab. ...4...4	67-100	82.5
6	48	mid oviduct	45	36	2-cell...11 4.....25	50-100	80.0
6	72	utero-tube junction	63	41	8.....5 16 .....12 mor. ...20 ab. 4	60-100	80.0
7	96	utero-tube junction or uterus	58	49	mor. ...10 bla. .....37 ab.....1	50-100	84.0
10	120	uterus	96	85	bla. .....80 ab. 5	50-100	88.5
4	144	uterus	42	5	later bla. 5	0- 30	8.0
2	168	uterus (implant)	22	0	0	0	0

* ab. is abnormal ova, mor. is morular, and bla. is blastula.

a defect of the method employed for the collection of ova, in which the rat ova were flushed out from the side of the uterus to the side of the oviduct. Therefore, the determination of the recovery rate of ova based on the number of corpora lutea should be regared as the problem necessary to be improved theoretically and technically. The recovery percent of the ova at the 144 and 168 hours after the observation of copulation plug was 8 percent and 0 percent, respectively. The lowered recovery percent was considered to be reasonable, because Alden (23) and Ishida (24) have made the histological study on the rat and showed that the fertilized rat ova at 144 hours after the observation of copulation plug has reached in the fold of the endometrial cell layer in the uterus. Since the fertilized rat ova has been implanted in the particular position of the uterus at 168 hours after the copulation, they were not flushed out from the uterus.

ii) Developmental stage of the rat ova.

The relation between the hours after mating and the developmental stage of the fertilized rat ova are given in Table 1. This results agreed with the those of histological and histochemical studies obtained by Alden (23) and Ishida (24).

All the rat ova obtained from the oviduct at 11 hours after the observation of copulation plug were of the one-cell stage. Of the rat ova collected from the oviduct at 24 hours after the copulation plug formation, 23 percent was of one-cell stage, 70 percent was of two cell stage and others were abnormal ova. At 48 hours after the observation of copulation plug formation, the fertilized ova developed into the stage from two-cell to four-cell stage. The percentage of each stage was 30 and 69.7 percent respectively.

At 72 hours after the copulation, the fertilized ova had developed into the eight-cell stage, 16-cell stage and morula stage, the recovery percent being 12, 30, and 50 percent respectively.

At four days after the copulation, the fertilized ova developed into the morula stage or the early blastura stage. The rate was 20 percent and 79 percent respectively. At five days after the observation of the presence of the numerous spermatozoa in the vaginal smear, all the fertilized ova reached at the later blastula stage.

It was concluded as a result of the observation of the rat ova during five days after copulation that the development of rat ova advanced slowly until 48 hours after the fertilization, and probably the cleavage of the fertilized ova occurred once for 24 hours, and the cleavage progressed relatively fast after 72 hours.

The unfertilized ova were collected from the oviduct of the females mated with the vigorous vasectomised male, and from the oviduct of the rat at 24 hours after the oestrus.

It was evidenced that the corona radiata of unfertilized ova is resolved by the semen in vivo or in vitro.

All the unfertilized ova collected from the oviduct of the females, which sacrified at 24 hours after oestrus without mating with the vasectomized male, had no corona radiata.

The fact that the corona radiata composing of the follicular cells had disappeared from the unfertilized ova suggests that such enzyme as the proteolitic emzyme by which corona radiata is resolved, must be secreted from the oviduct of the females at the oestrous period

iii) Appearance of the abnormal ova in the rat.

Of the ova collected from the genital tract of the females in the natural ovulation, some abnormal ova were occasionally discovered. They included, for instance, an ovum consisting of only the albumin layer or one having abnormal cytoplasma or one showing abnormal cleavage. The abnormal ova did not exceed more than 8 percent of the total ova collected.

Anyhow, it was apparent that the rat ova from the one-cell to the blastula stage could be obtained. By the method of the collection of the rat ova used in the present investigation, the ova without the epithelial cells and tissues of the genital tract of the pregnant female could be obtained.

It seems possible that this method can be applied to determine the ovulation number in the small animals, such as rat and mouse that have the oviduct of spiral form.

2. Oxygen consumption of the rat ova.

i) The changes in the oxygen consumption of the rat ova at the early stage of the development.

The oxygen consumption of the rat ovum per hour in the early stage of the development are given in Table 2. The oxygen consumption of the rat ova given in Table 2 represents the average value measured during one to three hours in the Cartesian diver.

As seen in Table 2, the oxygen consumption of the ova at the various stages of early development showed a marked deviation: the deviation between the minimum and the maximum value was from 30 to 55 percent.

The increased curve of the oxygen consumption of the ova at the early stage of the development are given in Fig. 1. As shown in Fig. 1, the oxygen consumption of the ova increased with the progress of the development and the S-shaped curve resulted. The oxygen consumption of the rat ovum in the one-cell, two-cell and four-cell stage was $0.56\times10^{-3}\mu l$, $0.85\times10^{-3}\mu l$, and $1.13\times10^{-3}\mu l$ respectively, showing a linear increase.

The oxygen consumption from the eight to 16 cell to morula stage increased slightly and the rate of the increase of the eight to 16 cell stage against the four-cell stage was only nine percent. The respiratory rate of the

morula stage was higher by 13 percent than that of eight to 16 cell stage. According to Boell and Nicholas (1) who studied on the oxygen consumption of the rat ova from the one-cell to eight to 16 cell stage the oxygen consumption of the rat ova increased in accordance with the progress of the development, and the rate of increase was about 32 percent. The value obtained in the present study were less than 23 percent as compared with that of Boell and Nicholas. The difference in each finding may be probably due to the different experimental conditions employed, such as strain of the rat, incubating medium, gas phase and temperature etc.

The increase rate of the oxygen consumption of the ova at the eight to 16 cell stage to morula stage was less than that of the ova at the one-cell to four-cell stage.

This results may be interpreted as follows: a) When the fertilized rat ova developed into the later stage after the 16-cell stage the shape of the developing ova became cubic and the area of the inner cell mass of embryo which contacted with the oxygen was less in degree than that of the ova at the four-cell stage. Accordingly the respiratory activity of the morula stage did not increase in proportion

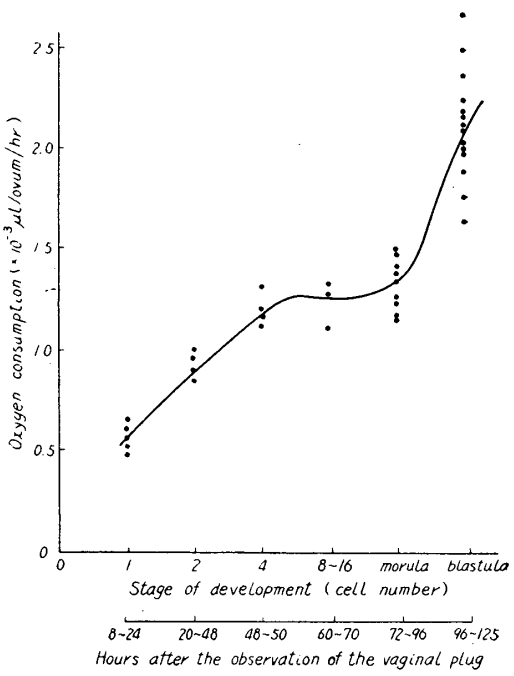

Fig. 1. Change in the oxygen consumption of the cleavage ova of the rat ova.

Table 2. The oxygen consumption of the fertilized and developing rat ova in vitro.

Developmental stage of ova	No. of experiment	Hours after observation of copulation plug		Oxygen uptake $\times 10^{-3}\mu l$/ovum/hr	
		Range	Average mean	Range	Average mean
1-cell stage	4	8-24	11	0.45-0.70	0.56
2-cell stage	5	20-48	35	0.773-1.023	0.85
4-cell stage	4	48-50	49	0.80-1.23	1.13
8-16-cell stage	5	60-70	65	0.80-1.35	1.23
morula	8	72-96	80	1.17-1.60	1.40
blastula	17	96-120	110	1.67-2.54	2.00

with the increased respiratory surface of the ova. On the other hand, the respiratory activity of the ova from the one-cell to four-cell stage increased in parallel with the increase of the surface of ova, because the fertilized ova developed with a plane form until four-cell stage. b) The concentration of the repiratory enzymes in the ova at the morula stage were the same as that of the four-cell stage.

The oxygen consumption of the ova at the blastula stage was markedly increased in comparison with that of the morula stage, the rate at the former stage was higher than that at the later stage by 43 percent, if medium contained 0.1 percent glucose.

It is of interest that the oxygen consumption of the ova at the blastula stage increased markedly closely related with the morphological change of the fertilized ova. They developed into the blastocyst at 120 hours after the mating and their form was elliptical and became somewhat larger in size.

Huber (25) has made the measurements of the volume of the rat ova a in the water displaced by wax reconstractions of fixed ova, and reported that the increase of the volume during from the one-cell stage to 16-cell stage was 35 percent.

But, it appears that the increase of the volume at the blastula stage is not due to the increase of the cytoplasma in the cells, because the rat ova at this stage differentiate into the inner cell mass and the trophoblasts and the center of the ova have the cavity.

Therefore, it may be suggested that the marked increase of the oxygen consumption at the blastula stage was originated from the increase of the respiratory enzyme in the cell of the blastocyst, and that the metabolic pattern changed into a specific pattern.

There is no literature on the oxygen consumption of the rat ova at the blastula stage. Fridhandler *et al.* (4) have made an investigation on the repiratory activity of the rabbit ova at the blastula stage and reported that oxygen consumption of the ova increased suddenly at that stage.

In the present investigation on the rat ova, almost a similar relation was found; namely, a marked increase in the oxygen consumption occurred at the blastula stage of the rat ova.

ii) Oxygen consumption per cell per hour in the developing rat ova.

From the basis of the oxygen consumption of the ovum between the one-cell to the four-cell stage, respiratory activity per cell was calculated.

The value at each stage was 0.56×10^{-3} μl, 0.43×10^{-3} μl, and 0.28×10^{-3} μl, respectively. As the cell number of the ova increased the oxygen consumption per cell per hour decreased gradually, because the size of the ova had not increased through these stages. From these results, it may be suggested that the metabolism of the rat ova expressed by tne oxygen consumption at the

cell level must be regulated by the rule of the metabolic rate.

iii) Q_{O_2} *of the developing rat ova.*

Studies on the volume change of the rat ova during the progress of the early development have been done by Huber (25) and Boell and Nicholas (26). Boell and Nicholas reported that the volume of the rat ova in the course of developing from the one-cell to the 16-cell stage had not increased, and that the wet weight of the ovum at these stages were 2.1×10^{-4}mg. Huber reported that the volume increased from 0.156 mμl at the one-cell stage to 0.211 mμl at the 16-cell stage.

On the basis of the results obtained by Boell *et al.*, the oxygen consumption of the rat ova at the various stages of the development we obtained was calculated and the Q_{O_2} values were obtained. They are shown in Table 3.

Table 3. Q_{O_2} values of the rat ova during the early stage of development.

Number of cells	Q_{O_2}	Number of cells	Q_{O_2}
1-cell	13.3	8-16 cell	29
2-cell	20.2	morula	33
4-cell	27.0		

As apparent from Table 3, the Q_{O_2} of the ova increased gradually from one-cell up to eight-16 cell stage and the results agreed with those obtained by Boell and Nicholas on the rat ova. They showed that the Q_{O_2} of the embryo on the eight day was 13.5 in the average, also Kleiber *et al.* (27) reported that the average value of Q_{O_2} on the 13 days old embryo was 7.2.

On the basis of their information and of the results obtained in the present investigation, it will be said that the Q_{O_2} of the rat ova increases gradually at the early stage of the development, namely until 10 days old, and there after may decrease during the periods of growth and differentiation.

The Q_{O_2} of the rat ova was higher by 3.0~4.5 times as much as that of the their liver. Thus it may be suggested that the metabolism of the ova at the early stage of the development makes the progress very effectively.

iv) *Oxygen consumption of the unfertilized rat ova.*

Oxygen consumption of the unfertilized and fertilized ova at the one-cell stage are given in Fig. 2 and in Table 4. As apparent from Fig. 2, there is no difference in the oxygen consumption between the unfertilized and the fertilized ova. The oxygen consumption of the unfertilized ovum was 0.53×10^{-3} μl/hr, while the value of the fertilized ovum at eight hours after the detection of the many spermatozoa in the vaginal smear was 0.54×10^{-3} μl/hr. The oxygen consumptions of the unfertilized ova were not depressed completely when they were left in vivo for 70 hours after the oestrus.

Table 4. Oxygen consumption of the unfertilized rat ova at the various time after the oestrus.

	No. of determination	Hours after the observation of copulation plug		Oxygen consumption $\times 10^{-3}\mu l$/ovum/hr	
		Range	Average	Range	Average
Fertilized	4	8-24	11	0.45-0.70	0.56
Unfertilized	3	10-14	12	0.40-0.68	0.50
Unfertilized	3	20-24	21.6	0.50-0.79	0.61
Unfertilized	3	60-78	70	0.31-0.71	0.49

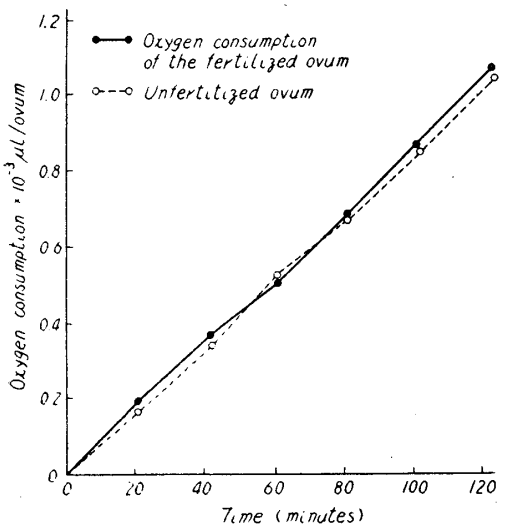

Fig. 2. Oxygen consumption of unfertilized and the fertilized ova at the one cell stage.

Fridhandler *et al.* (2) have reported that the oxygen consumption of the fertilized and unfertilized rabbit ova was the same in both and that the unfertilized ova has not changed when they were left up to 56.5 hours in vivo. In the present experiment, it was about the same as their results.

In the rat, the fertilized ova had not developed up to the first cleavage at eight to-11 hours after the observation of the spaermatozoa in the vaginal smear. Thus this fact may suggest that the union of the female pronuclei and the male pronuclei, *e.g.* syngamy, does not come to an end, and fertilization is still incomplate. Also in the rabbit, Fridhandler *et al.* have reported that the oxygen consumption on the activated ova increased at 29.1 hours after the activating treatment on the ova.

Therefore, it may be said that oxygen consumption of the rat ova must be stimulated by the complete union of the both pronuclei.

v) Effects of several buffers on the oxygen consumption of the blastocyst of the rat.

Oxygen consumption of the ova at the blastula stage measured with several buffer (pH=7.4), as Krebs-Ringer-Phosphate Buffer (K.R.P), $CaCl_2$-free K.R.P., $CaCl_2$-free K.R.P. containing of 0.1 percent glucose, $CaCl_2$-free K.R.P. diluted with the auto-serum (1:1) and serum are shown in Table 5.

As apparent in Table 5, when the $CaCl_2$-free K.R.P.+0.1 percent glucose and $CaCl_2$-free K.R.P.+the auto-serum (1:1) were used as the medium, oxygen

consumption of the ova at the blastula stage was nearly 2.0×10^{-3} μl per ovum and these activities did not differ.

Also, when $CaCl_2$-free K.R.P. were used as the medium, the oxygen con-

Table 5. Effects of several buffers on the oxygen consumption of the rat ova at the blastura stage.

Buffer	No. of determination	Oxygen consumption $\times10^{-3}\mu l$/ovum/hr	
		Range	Average
K.R.P.*	5	0.40-0.99	0.79
$CaCl_2$-free K.R.P.	5	0.59-0.91	0.81
$CaCl_2$-free K.R.P.+0.1% glucose	17	1.67-2.54	2.00
$CaCl_2$-free K.R.P.+serum (1 : 1)	3	1.55-2.25	1.93
Serum	1	1.48	1.48

* Krebs-Ringer-Phosphate Buffer (pH=7.4).

sumption of the rat blastocyst did not differ in both medium. It may indicate that Ca^{++} ion has no influence on the oxygen consumption of the ova at the blastula stage. On the basis of the facts mentioned above, it may be said that the available medium for the measurement of the oxygen consumption of the ova developed to the blastula stage is $CaCl_2$-free K.R.P. buffer contained such substrate as glucose or nutrients in serum. The metabolic pattern of the rat ova must be changed at the blastula stage, because the respiratory activity was 2.4 fold higher when the substrate (glucose or serum) was added to the $CaCl_2$-freee K.R.P., than that of the value obtained in the case of no substrate.

Summary

We dealt with the improvement of the ova collection technique and the respiratory activity of the rat ova at the early stage of the development. The respiratory activity was measured by the Cartesian diver method modified slightly by authors. The results are summarized as follows.

1. In the present study, the rat ova were collected by the method in which the contents of the oviducts and uterus were flushed out into the Petri dish with the buffer and they were used for the measurement of the oxygen consumption. In this method, recovery percent, *e.g.* the ratio between the number of the collected ova and the fresh corpora lutea (in the overy) was 83.2 percent on an average.

2. As the development of the rat ova their oxygen consumption increased gradually.

3. The oxygen consumption of the rat ova from the one-cell stage up to the four-cell stage increased linearly, while it showed a sudden increase at the blastula stage.

4. There was no difference in the repiratory activity between the unfertilized and fertilized rat ova at the one-cell stage. The oxygen consumption of the unfertilized ova did not become depressed until 70 hours after the oestrus stage in vivo.

5. The oxygen consumptions of the ova from the one-cell stage to the 16-cell stage were represented as Q_{O_2}. The Q_{O_2} value of the ova at the one-cell stage was 13.3, and the value was higher by 3.0-4.5 fold than that of their liver.

6. The metabolism of the rat ova at the blastura stage responded to the substrate. When the glucose or serum was added to the $CaCl_2$-free K.R.P., therefore, the oxygen consumption of the rat blastocyst increased two-folds in comparison with the value obtained in the case of no substrate.

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