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Incorporation of ^{14}C-L-Leucine in Rat Eggs of Preimplantation Stage II. Effects of Addition of Glucose

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Summary

The incorporation of ^{14}C-L-Leucine was examined of rat eggs at three stages. The incorporation of ^{14}C-L-Leucine increased in 1-cell (unfertilized) and 2-cell eggs after 0.5 hr. of incubation and in the blastocyst stage after 3 hr. of incubation ($p<0.05$).

The addition of Actinomycin D has inhibited the incorporation of ^{14}C-L-Leucine in eggs of various stages. It seems to indicate that the eggs synthesize a neo-protein from a free amino acid and the trichloroacetic acid insoluble fraction is a protein synthesized. The addition of glucose as an energy source had no effect on incorporation of ^{14}C-L-Leucine in 1-cell and 2-cell embryos, but increased the incorporation in the early blastocyst stage.

It has been reported that there is a rapid increase in the incorporation of labelled amino acids into protein shortly after fertilization or parthenogenetic activation of sea urchin eggs[1-4].

However, there is little information on the metabolism of amino acid in mammalian eggs. This is due to the measurement difficulty. One of the reasons was a difficulty in obtaining an adequate number of mammalian eggs for measurement. However, this problem has been solved by the induction of superovulation. Also the measurement of incorporation value was possible using only a comparatively few eggs because of the liquid scintillation techniques.

We have previously studied the relationship between the incorporation of amino acid, the number of eggs, and the time of incubation[5]. The purposes of the present investigation were (a) to examine the incorporation of ^{14}C-L-Leucine in various stages of the eggs using the methods reported previously (b) to ascertain whether the synthesis of protein occurs in early development stage of the eggs of the rat by the application of Actinomycin D. (c) to exmaine the effects of addition of glucose to the medium as an energy source on the incorporation of L-Leucine in the early embryonic stages.

Materials and Methods

All animals used were female rats of the Wistar strain. The eggs used in these experiments were 1-cell, 2-cell and early blastocyst: (1) 1-cell (unfertilized) eggs were obtained from the immature female rats superovulated by the injection of 30 iu pregnant mare's serum (PMS) followed 48 hrs. later with an injection of 10 iu of human chorionic gonadotropin (HCG)[6]. The eggs were picked by a pin from ampulla on a watchglass at 28 to 29 hrs. after the injection of HCG. For recovery of eggs, chymotrypsin was added to a watchglass (final concentration 0.008%). The granulocytes disappeared within ten minutes at room temperature. Following digestion, the eggs were washed several times by transferring in Ca-free Krebs-Ringer phosphate (K.R.P.) (pH 7.4) for elimination of chymotrypsin. (2) 2-cell eggs were obtained from virgin female rats. Each animal was injected intramuscularly with 50 iu of PMS at early diestrus, and 48 hr. later was injected intramuscularly with 50 iu of HCG[7]. Immediately after HCG injection, each female was placed with a fertile male and mating was confirmed by the presence of a vaginal plug the following morning (Day 1 of pregnancy). The eggs were flushed from the fallopian tubes of mated females on Day 2 of pregnancy, approximately 49 hrs. after the HCG injection. (3) Early blastocysts were obtained from adult female rats. The female in proestrus was caged overnight with a fertile male. The mated females were killed on Day 5 of pregnancy, at 102 hrs. after the estimated time of fertilization. The blastocysts were flushed from the uterus.

The basic medium used in the study was K.R.P. (pH 7.4). The same solution was used for all manipulations.

The eggs collected in a watchglass were examined under a microscope and the normal eggs were transfered into 0.3 ml of solution in a centrifuge tube with a capillary pipett. The number of embryos used for measurement were 50 per one sample[5]. 1 to 1.5 hours was taken for the manipulation. After preincubation at 37.5°C for 10 minutes, 0.5 μci/ml universal labelled ^{14}C-L-Leucine (spec. act. 0.396 mci/ml) was added. These sample were incubated for 0, 0.5, 1, 2 and 3 hrs.

In the case of addition of 0.1 ml Actinomycine D or glucose, the eggs were collected in 0.2 ml of solution, following addition of 0.5 μci/ml of ^{14}C-L-Leucine at incubation. The final concentration of Actinomycin D was 10 μg to 0.25 μg which is generally used for mammalian cells[8]. 10^{-3}M or 10^{-2}M concentrations of glucose were used[9,10].

After each incubation time, the reaction was stopped by addition of cold trichloroacetic acid (TCA) to a final concentration of 5 percent. Then following Millipore filtration (scwp 8 μ), the acid insoluble material was washed with 5 percent TCA. The filtrate was transfered to the vials for determination of radioactivity by liquid scintillation counter (Packard Tri-Carb), using 1,000 ml

of toluene containing 4 g 2,5-diphyenyloxazole (PPO) and 300 mg 1, 4-bis-[2(4-Methyl-5-Phenyloxazolyl)]-Benzene (dimethyl POPOP) as a scintillator. The real incorporation value was estimated the measured value minus the 0-time value. The results were treated statistically by the method of variance analysis. The viability of the eggs after incubation was estimated by its staining affinity in nigrosin (0.03%)[11].

Results

The incorporation of ^{14}C-L-Leucine: — The relation between incubation time and incorporation of ^{14}C-L-Leucine in each stage of egg development is shown in Table 1 and Fig. 1. The values for 1-cell and 2-cell eggs were almost similar in each incubation time ($P>0.1$). As shown in Fig. 1, the incorporation of L-Leucine in 1-cell and 2-cell eggs increased with 1 hr. of incubation ($P<0.05$).

On the other hand, early blastocyst showed a rapid increase of incorporation of L-Leucine after 2 to 3 hrs. of incubation ($P<0.05$). The difference between 1 and 2 hrs. was not significant statistically ($P>0.1$). The results from staining affinity of nigrosin have proved that the eggs were survived during these incubation times.

The effects of Actinomycin D on the incorporation of ^{14}C-L-Leucine: — The inhibitory dose of Actinomycin D in incorporation of L-Leucine was investigated in 1-cell stage eggs. The incorporation of ^{14}C-L-Leucine in eggs was completely inhibited by each dose of Actinomycin D, as shown in Table 2.

Whether or not incorporation of ^{14}C-L-Leucine is inhibited by addition of Actinomycin D 1 μg in eggs of various stages of development has been examined 1-cell and 2-cell eggs were measured in only ^{14}C-L-Luecine, but the early blastocysts were measured after addition of 10^{-2}M glucose. The incorporation of ^{14}C-L-Leucine was completely inhibitied, as shown in Table 3. From the data obtained in addition of Actinomycin D, it seems to indicate that eggs of various stages can synthesize a

TABLE 1. *Incorporation of ^{14}C-L-Leucine in Rat Egg*
(Mean±S.E.) (CPM/egg)

Stage of egg \ Incubation time (hr.)	0.5	1	2	3
1-cell	36.0± 8.2 (9)	64.9± 8.0 (8)	70.0±12.2 (8)	—
2-cell	60.8±10.7 (8)	84.1±14.9 (8)	83.1±10.1 (9)	—
Early Blastocyst	—	8.2± 5.5 (11)	37.4±13.0 (8)	136.7±13.3 (8)

( ): No. of observation.

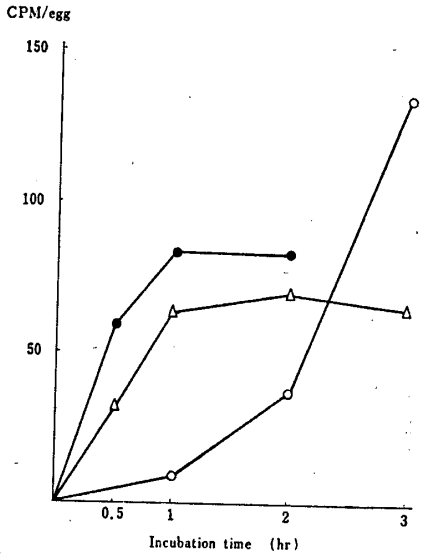

FIG. 1. Incorporation of ^{14}C-L-Leucine of each stage of egg in each time of incubation. (CPM/egg)

TABLE 2. *Effects of Various Doses of Actinomycin D on Incorporation of ^{14}C-L-Leucine* (Mean ±S.E.) (CPM/egg)

Incubation time (hr.) \ Dose of Actinomycin D	10 μg	1 μg	0.5 μg	0.25 μg
0	8.3±2.3 (6)	15.4±2.8 (9)	12.3±1.0 (6)	17.7±3.9 (4)
1	7.3±1.3 (6)	10.2±1.1 (9)	11.6±0.8 (6)	14.0±3.7 (4)

( ): No. of observation.

neo-protein from a free amino acid. It is possible that the incorporation of ^{14}C-L-Leucine into the TCA insoluble fraction is protein synthesis.

Effects of glucose addition on the incorporation of ^{14}C-L-Leucine: —

a) 10^{-3}M glucose: The effects of 10^{-3} M glucose addition on the incorporation of ^{14}C-L-Leucine were investigated in 1-cell and 2-cell eggs. These results are shown in Table 4. The values of 1-cell and 2-cell eggs at 0.5 hr. of incubation were not significant statistically ($P>0.1$) as comparing with only ^{14}C-L-Leucine. Thus, the incorporation of ^{14}C-L-Leucine in 1-cell and 2-cell eggs was not affected by addition of 10^{-3}M glucose.

TABLE 3. *Effects of Actinomycin D* (1 μg) *on Incorporation of ^{14}C-L-Leucine* (Mean±S.E.) (CPM/egg)

Stage of egg \ Incubation time (hr.)	0	1
1-cell	15.4±2.8 (9)	10.2±1.1 (9)
2-cell	12.5±2.4 (6)	9.4±1.9 (6)
Early Blastocyst	9.6±1.8 (6)	10.4±1.6 (6)

( ): No. of observation.

TABLE 4. *Effects of $10^{-3}M$ Glucose Addition on Incorporation of ^{14}C-L-Leucine* (Mean±S.E.) (CPM/egg)

Stage of gge \ Incubation time (hr)	0.5	1
1-cell	39.5± 6.4 (5)	50.0±8.6 (5)
2-cell	38.2±11.7 (6)	—

( ): No. of observation.

b) 10^{-2} M glucose: The results obtained here are shown in Table 5. Significant increases of incorporation were observed in 1-cell, 2-cell and early blastocyst stage at 1 hr. of incubation compared with that of 0.5 hr. ($P<0.005$). 1-cell and 2-cell stages added with glucose were not statistically different compared with only ^{14}C-L-Leucine at 0.5 and 1 hrs. of incubation, respectively ($P>0.05$). The incorporation of ^{14}C-L-Leucine in 1-cell and 2-cell eggs were not affected by addi-

TABLE 5. *Effects of $10^{-2}M$ Glucose Addition on Incorporation of ^{14}C-L-Leucine* (Mean±S.E.) (CPM/egg)

Stage of egg \ Incubation time (hr.)	0.5	1
1-cell	2.0±8.7 (7)	36.2±8.4* (8)
2-cell	21.5±8.5 (7)	67.7±7.7* (9)
Early Blastocyst	24.0±2.4 (6)	64.2±9.8* (8)

( ): No. of observation.
*: $P<0.05$

tion of 10^{-2}M glucose. On the other hand, early blastocyst showed a significant increase of incorporation when 10^{-2}M glucose was added to the incubation medium, comparing with only ^{14}C-L-Leucine at 1 hr. of incubation ($P<0.05$), as shown in Fig. 2.

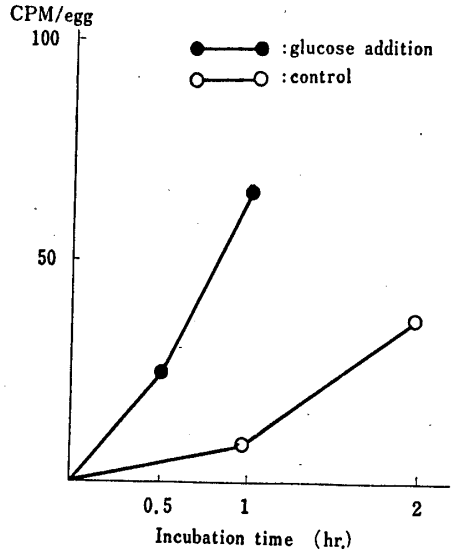

FIG. 2. Effect of 10^{-2}M glucose addition on incorporation of ^{14}C-L-Leucine in early blastocyst of rat.

Discussion

It seemed that the eggs of each stage showed a different pattern of incorporation of ^{14}C-L-Leucine added in Krebs-Ringer phosphate (pH 7.4). Mintz[12] has observed by the autoradiographical method that protein synthesis from ^{3}H-Leucine occurs in the fertilized mouse egg before cleavage, though at low levels in unfertilized eggs. He also noted that the increase of protein synthesis near the time of the third cleavage is significant, and is more pronounced at blastocyst. This report is similar to our result that the embryo during early development has a different pattern of incorporation at various cleaving stages. But, this is not in agreement with the view that the unfertilized eggs have a low level of protein synthesis. Recently, Tyler[13] has shown that protein synthesis occurs in the ripe unfertilzied eggs of sea urchins. It may be suggested that the pattern of protein synthesis at developmental stages is different between species.

Actinomycin D is known to block the synthesis of protein[14]. In the results obtained, the incorporation of ^{14}C-L-Leucine in eggs was completely inhibited by addition of Actinomycin D. It is suggested that the isotope incorporated into the TCA insoluble fraction indicates a protein synthesized from the L-Leucine.

There is a large glycogen store in the early preimplantation stages of the mouse egg and the stored glycogen is apparently not utilized before the morula stage[15].

However, the glycogen content of the egg begins to decrease with the initiation of blastocyst formation. Brinster[10] has examined the carbon dioxide production from labelled glucose in mouse eggs, and noted a gradual increase during the developmental stages and a maximum value at the late blastocyst stage.

The addition of glucose had no affect on the incorporation of ^{14}C-L-Leucine in 1-cell and 2-cell eggs, but increased the incoporation at early blastocyst. It may be suggested that the activity of incorporation does not reduce when glucose is added as an energy source at blastocyst formation.

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