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Effects of Progesterone on the Macromolecular Syntheses in Mouse Preimplantation Embryos *in Vitro*

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프로제스트론이 培養中인 생쥐 初期胚兒의 高分子化合物合成에 미치는 影響에 관하여

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適 要

Progesterone은 배양중인 생쥐 초기배아의 난할을 억제하는 효과를 나타내고 있는데 이의 기작을 밝히기 위하여 이 호르몬이 배아 세포들의 각종 대사작용에 미치는 영향을 조사하여 다음과 같은 결과를 얻었다.

1. Progesterone은 배아의 아미노산 흡수능 (uptake)을 증가시키었으나 동화능 (incorporation)을 현저히 감소시켰다.

2. Progesterone은 핵산 전구물질들 (uridine과 thymidine)의 흡수능과 동화능을 모두 저하시켰다.

본 실험의 결과로 미루어 Progesterone은 배아 세포들의 단백질, RNA 그리고 DNA 등의 고분자화합물의 합성을 저해하여 배아의 난할을 억제하는 것으로 보이며 배아 세포들의 투과성의 변화와는 직접 관련이 없는 것으로 사료된다.

INTRODUCTION

Since Whitten (1957) reported that progesterone in the culture medium has a deleterious effect on the cleavage of the early mouse embryos *in vitro*, several investigators have studied the effect of steroids on the development of preimplantation embryos in mammals both *in vivo* and *in vitro* (Daniel, 1964; Daniel and Levy, 1964; Kirkpatrick, 1972; Allen and Foote, 1973; Kwon and Chung, 1975). Daniel (1964) found that a direct exposure of steroids to rabbit embryos

brings about the inhibition of cleavage or delay in development. Pratt and Daniel (1966) also showed that progesterone is more effective than any other steroids in this action, and the resistance of the embryos to progesterone increases as the embryos develop. However, the action mechanism of progesterone in mammalian embryos *in vitro* has not been fully understood yet.

From their finding that rabbit embryos cleaved normally in a medium containing progesterone if excess amino acids or serum were present in the medium, Daniel and Levy (1964) postulated that progesterone in the medium changes the permeability of egg membrane and thus restricts the transport of the nutrients such as amino acids which are essential for the embryos to cleave. Kirkpatrick (1972), however, showed that progesterone did not interfere with the uptake of energy sources such as lactate in mouse embryonic cells. Recently, Cho and Kwon (1976) observed by autoradiography that the protein synthesis of two-cell embryos is suppressed under the influence of progesterone. So far no one has yet attempted to determine the various metabolic changes of the embryos by the hormone, which seem to be closely related to the hormone action *in vitro*.

The present experiments were designed (1) to determine the change of the transport systems of various substrates on embryos under the influence of progesterone, (2) to examine the macromolecular synthetic activity of the embryos in the presence of progesterone, and (3) to investigate the permeability of progesterone itself to the embryos in order to elucidate the action mechanism of progesterone on mammalian embryos *in vitro*.

MATERIALS AND METHODS

Collection of embryos

A random-bred A-strain mice were used throughout these investigation. The animals were provided from the Experimental Animal Farm, Seoul National University.

To obtain embryos in a sufficient quantity, two-month old female mice were superovulated by injection of each 5 I.U. pregnant mare's serum (PMS, Sigma) and human chorionic gonadotrophin (HCG, Sigma) 48 hours apart. The female mice given HCG injection were placed with fertile males and the female was checked for vaginal plug on following morning to confirm copulation. Two-cell embryos were flushed out from the fallopian tubes of mated female mice at 44-48 hours, and morulae were released from the isthmic portion of the oviducts and the upper part of the uteri at 72-76 hours. Blastocysts were flushed out from the uteri at 96-100 hours after the injection of HCG in a drop of basic medium in a sterile Petri dish.

Culture of embryos

Cultivation of the embryos with progesterone was carried out by applying the micro tube culture system, because the hormone is oil soluble (Cho, 1974). Incubation of the embryos was carried out in 5 μl of culture medium set in the middle part of a micro tube in an atmosphere of 5% CO_2 in fully moistened air at 37°C. The detailed procedures for this culture technique are described elsewhere (Cho, 1974; Cho et al., 1974).

The basic medium used for washing and recovering of the embryos was a slightly modified standard egg culture medium (SECM) which is a modified Krebs-Ringer bicarbonate salt solution (Biggers et al., 1971) and the culture medium was supplemented with 0.4% bovine serum albumin (BSA). Progesterone was first dissolved in absolute ethanol and diluted concentration and the final concentration of ethanol in the medium was not allowed to exceed 1%.

Radioactive precursors with the listed specifications were obtained from the following sources; ^{14}C-L-glutamine (about 230 mCi/mmole in 2.5 ml water) from New England Nuclear Corp.; L-(4,5-^{3}H) leucine (50 Ci/mmole in 2% ethanol), (6-^{3}H) uridine (14 Ci/mmole in 2% ethanol) and ^{3}H-progesterone (49 Ci/mmole in benzene solution) from Amersham/Searle Corp.; (methyl-^{3}H) thymidine 40-60 Ci/mmole in 2% ethanol) from Schwartz/Mann. A determined volume of each radioactive precursor was directly added to the culture medium to desired concentration of label.

Preparation for scintillation counting

At the end of the culture period, the embryos were collected and rapidly washed three times by sequential transfer to 2 ml of basic medium. They were then transferred to a microcentrifuge tube containing 50 μl of H_2O plus 1 mg of BSA which acted as cold carrier.

The tubes were kept at −20°C until all stages were collected for each replicate. The samples were than frozen and thawed three times. Five μl of the last wash was taken as a blank for the embryo sample. Fifty μl of 13% cold trichloroacetic acid (TCA) was added to the tubes, the contents were mixed, and the tubes were stored for over 20 minutes, and then centrifuged for two minutes in a Microfuge (Beckman). The TCA precipitate was then resuspended in 25 μl of 5% TCA, the tubes were vortexed gently, and then stored for 20 minutes. The tubes were centrifuged again for two minutes in a Microfuge and the supernatant fluid was removed and pooled with the soluble fraction.

The precipitate was dissolved in 100 μl of 10% KOH for 30 minutes and was then transferred to a scintillation vial (insoluble fraction), and to which were added 10 ml of scintillation fluid which contained 0.5% (W/V), 2,5, diphenylnyloxazole (PPO) and 0.01% (W/V), 1,4,-bis (2-(-phenyloxazolyl))-benzene (POPOP) in ethanol-toluene (1 : 3) mixture. The radioactivity was determined using a

Packard Tri-carb scintillation spectrometer.

RESULTS

The syntheses of protein, RNA and DNA were examined by measuring of the incorporation rate of their labelled precursors, i.e., leucine, uridine and thymidine, respectively, and the permeability was determined by measuring of the total uptake of the precursors into the embryonic cells in the presence of progesterone in the culture medium. On the basis of the previous studies (Kwon and Chung, 1975), the hormone concentrations were ranged from 2.5 μg/ml to 40 μg/ml, and at the latter concentration the cleavage was completely blocked.

1. Protein synthesis

Morulae were preincubated in the presence of progesterone (2.5 to 40 μg/ml) for two hours and then ^{3}H-leucine (10 μCi/ml) was added for an additional two hours of incubation. The results are shown in Fig. 1. Progesterone at the dose of 20 μg/ml in the medium stimulated the uptake but suppressed the incorporation of ^{3}H-leucine by morulae. Blastocysts took up ^{3}H-leucine in the presence of

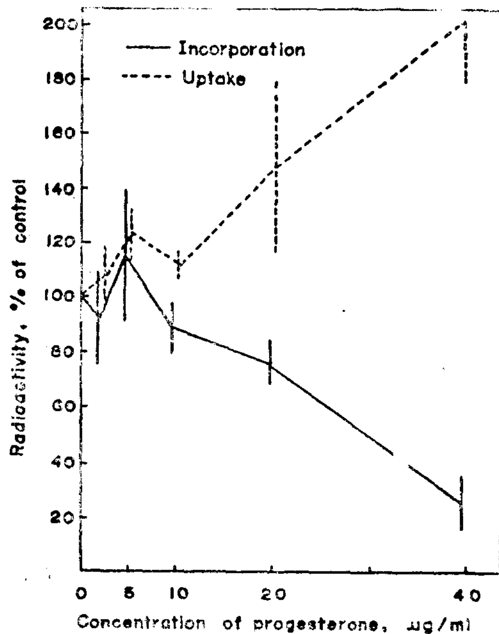


Fig. 1. Effects of progesterone on the uptake and incorporation of ^{3}H-leucine in early mouse morulae *in vitro*. The morulae were cultured in the presence of progesterone (2.5-40 μg/ml) for two hours and then labelled for two hours with ^{3}H-leucine (10 μCi/ml).

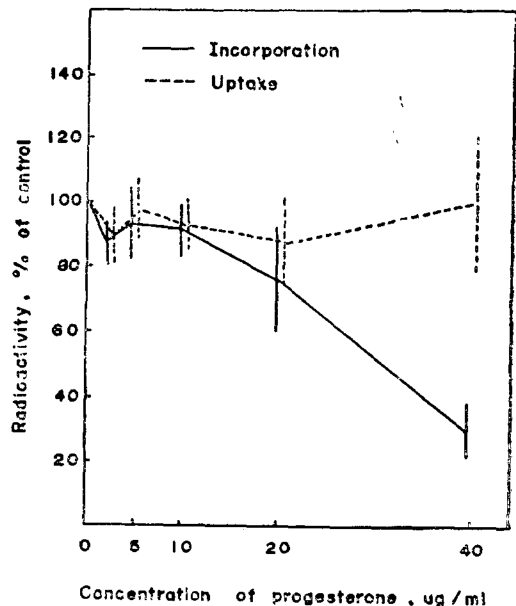


Fig. 2. Effects of progesteroneon on the uptake and incorporation of ^{3}H-leucine in mouse blastocysts *in vitro*. The blastocysts were cultured in the presence of progesternoe (2.5—40 μg/ml) for two hours and then labelled for two hours with ^{3}H-leucine (10 μCi/ml).

progesterone even at such a high dose as 40 μg/ml, but its incorporation was markedly decreased to 30% of control value (Fig. 2).

The above results show that transport of amino acid into the embryonic cells takes place as usual even in the presence of progesterone, but its incorporation into the protein is suppressed. These results indicate that progesterone does not affect the permability of the membrane to amino acid but does affect the protein synthesis. It was found that blastocysts are more resistant to exogeneous progesterone than morulae *in vitro.*

2. RNA synthesis

To determine the effects of progesterone upon RNA synthesis mouse at different stages of development were explanted into culture medium and labelled with ^{3}H-uridine in the absence or presence of progesterone. When morulae were cultured for four hours with ^{3}H-uridine (20 μCi/ml) in the presence of various concentrations of progesterone, both the uptake and incorporation of ^{3}H-uridine by the morulae were severely affected by the hormone at the concentration of 10 μg/ml, and they were reduced to about 20% of control levels at 20 μg/ml (Fig. 3).

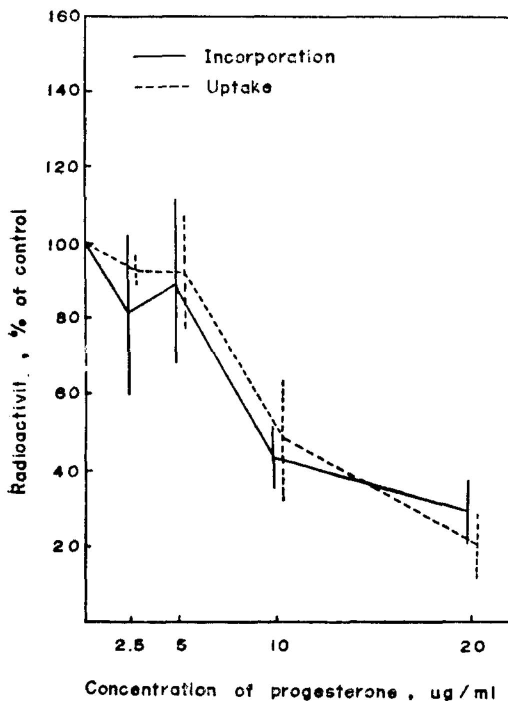


Fig. 3. Suppression of the uptake and incorporation of ^{3}H-uridine by progesterone in early mouse morulae *in vitro.* The morulae were labelled for four hours with ^{3}H-uridine (20 μCi/ml) in the absence or presence of progesterone (2.5—20 μg/ml).

In papallel experiments, late mouse morulae and blastocysts were also cultured on the same condition and the data obtained were plotted in Figs 4 and 5. From the figures, it is seen that the addition of progesterone to the culture medium produced a significant dose dependent suppression of the uptake and incorporation of ^{3}H-uridine by the embryos. Comparing the above three results (Figs. 3, 4 and 5), it is seen that there exists a difference in the sensitivity to progesterone among embryos of different stages. For example, at the early morula stage, RNA synthesis was reduced in the presence of progesterone (20 μg/ml) to about 25% of control, while the reduction was 40% of control at late morula stage and

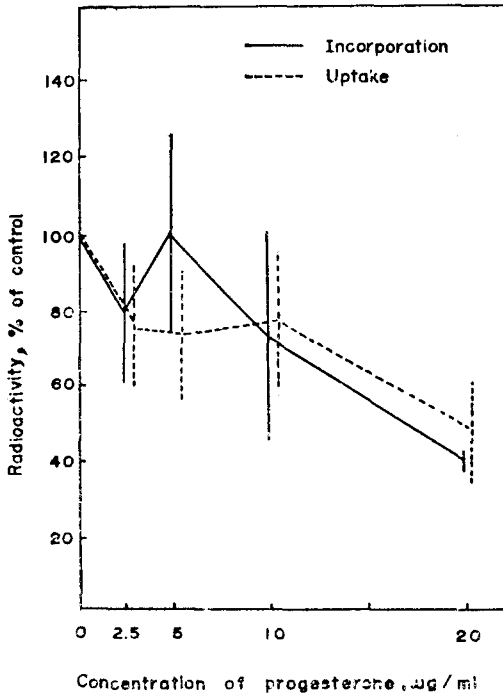
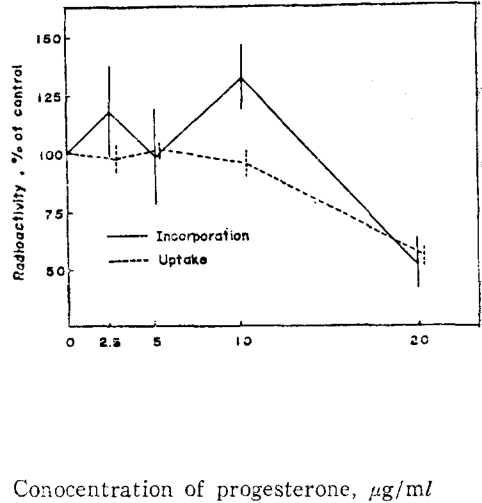


Fig. 4. Suppression of the uptake and incorporation of ³H-uridine by progesterone in late mouse morulae *in vitro*. The morulae were incubated for four hours in the presence of progesterone (2.5—2.0 μg/ml) and ³H-uridine (20 μCi/ml).



Conocentration of progesterone, μg/m*l*

Fig. 5. Effects of progesterone on the uptake and incorporation of ^{3}H-uridine in mouse blastocysts *in vitro*. The blastocysts were incubated for four hours in the presence of progesterone (2.5—20 μg/ml) and ^{3}H-uridine (20 μCi/ml).

55% at blastocyst stage. This differential sensitivity to the progesterone indicates a higher resistance as the early embryos progressed in development.

In contrast to the amino acid transport, uridine transport into the embryonic cells was remarkably influenced by the exogeneous progesterone, and its incorporation into the RNA molecules was also affected as much as its transport. From the results, it is inferred that the inhibition of RNA synthesis may be caused by the presence of progesterone which might regulate the uptake of uridine.

3. DNA synthesis

Mouse morulae or blastocysts were preincubated with progesterone (2.5 to 40 μg/ml) for two hours, and then with ^{3}H-thymidine (20 μCi/ml) for an additional four hours of incubation. The data are plotted in Figs. 6 and 7. The patterns of the uptake and incorporation of ^{3}H-thymidine are very similar to those of uridine, showing a dose dependent suppression of DNA synthesis and of permeability of DNA precursors. However, the uptake of ^{3}H-thymidine into the

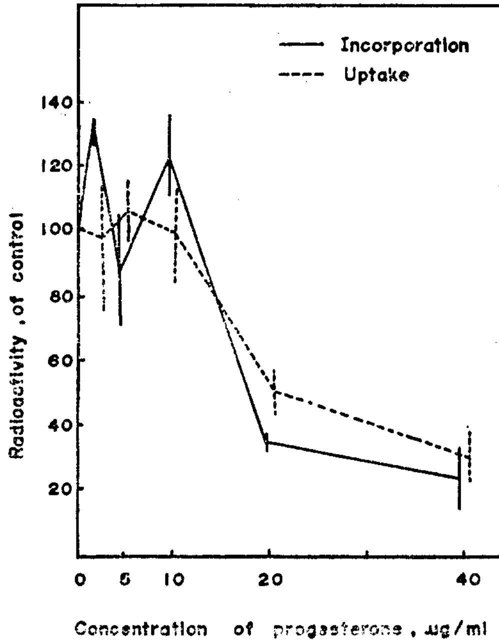


Fig. 6. Suppression of the uptake and incorporation of ^{3}H-thymidine by progesterone in mouse morulae *in vitro*. The morulae were cultured in the presence of progesterone (2.5—40 μg/ml) for two hours and then labelled with ^{3}H-thymicine (20 μCi/ml) for four hours.

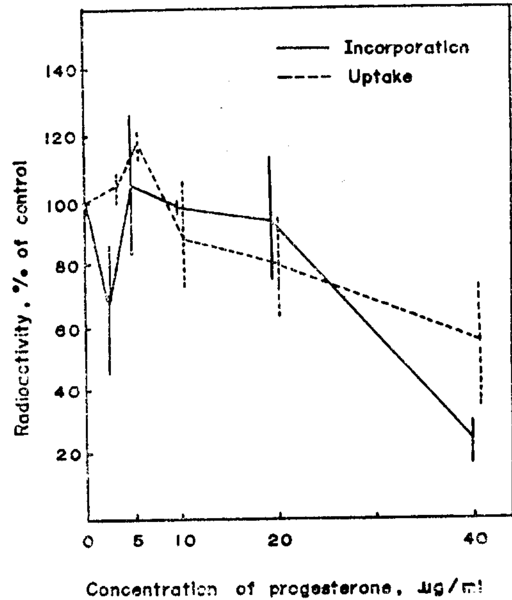


Fig. 7. Effects of progesterone on the uptake and incorporation of ^{3}H-thymidine in mouse blastocysts *in vitro*. The blastocysts were cultured in the presence of progesterone (2.5—40 μg/ml) for two hours and then labelled with ^{3}H-thymidine (20 μCi/ml) for four hours.

embryonic cells is less suppressed by the exogeneous progesterone than uridine is.

The incorporation rate of ^{3}H-thymidine into DNA by morulae also decreased significantly at the concentration of 20 μg/ml of progesterone as can be seen in Fig. 6. In blastocysts, too, progesterone significantly suppressed the DNA synthesis and ^{3}H-thymidine uptake at the dose of 40 μg/ml (Fig. 7).

From the observation that RNA synthesis in blastocyst was affected by even such a low dose of 20 μg/ml of progesterone where practically no suppression of DNA synthesis was observed, it is apparent that DNA synthesis system is less susceptible to progesterone than that of RNA in mouse embryos.

4. Uptake patterns in the various stages of the embryos

To examine the uptake patterns of precursors by follicular oocytes and embryos at various stages from one cell (zygote) to blastocysts, they were labelled with ^{14}C-glutamine (5 μCi/ml) for two hours or with ^{3}H-uridine (20 μCi/ml) for four hours and the total uptake of the precursors was measured as

described before. The results are summarized in Table 1. These results indicate that oocytes and the late morulae take up glutamine actively while the embryos between these two stages do less.

The uridine uptake is very low in oocytes and embryos up to early morula, then drastically increases in the late morula.

In general, one-cell and two-cell embryos are nearly inert in the uptake of glutamine and uridine, but beyond that stage, there is a gradual increase and most drastic uptake occurs at late morula stage.

5. The uptake pattern of progeaterone in mouse embryos *in vitro*

Oocytes or embryos were labelled with ^{3}H-progesterone (50 μCi/ml, 0.32 μg/ml) for two hours, in order to determine whether the embryonic cells take up the progesterone and to evaluate the effect of exogeneous concentration of progesterone. The concentration of progesterone in the medium was adjusted with cold progesterone to 0.32, 5 or 20 μg/ml. The data obtained are expressed as

Table 1. Developmental pattern of glutamine and uridine utake in mousee mbryos *in vitro*

Developmental stages	2hr culture	4hr culture
	^{14}C-Glutamine Uptake	^{3}H-Uridine Uptake
Oocyte	780±40[a]	36± 12
One cell	118± 6	36± 6
Two cell	128± 4	54± 6
Early Morula	366± 8	372± 100
Late Morula	704±50	3400±1196
Blastocyst	1334±50	3314± 634

a. The counts/min per 20 embryos±S.E

Tabel 2. Pattern of progesterone uptake by mouse embryos *in vitro*

A. Concentration of progesterone (μg/ml)	Developmental stages		
	Oocytes	Morulae	Blastocysts
0.32	120.4±16.5[a]	239.4±25.8	389.6
5	91.8± 8.2	191.6±46.4	481.6
20	115.8±51.8	228.8±43.0	429.0

B. Culture hour	Oocytes	Morulae
0.5	90.8±23.2	110.4±23.0
1	112.2± 8.6	187.8±28.8
2	126.0±15.6	164.0±39.4

a. The counts/min per 20 oocytes±S.E.

counts/min per 20 embryos and the results are summarized in Table 2. Table 2A shows that all the embryos took up progesterone from the medium and the uptake increased as development progressed. Morulae took up ^{3}H-progesterone about two-fold of oocyte value, and blastocyst, about four-fold. The concentrations of progesterone (0.32, 5 or 20 $\mu g/ml$) in the medium did not affect the uptake of labelled progesterone (Table 2A). As Table 2B shows, the uptake of progesterone into the egg reached maximum within one hour in culture.

DISCUSSION

Even though many investigators have studied on the effects of progestogen in early embryogenesis of mammals, there are few reports concerned with the metabolism of embryos treated with the hormones. In contrast to the results of Pratt and Daniel (1966), the present results indicate that the syntheses of macromolecules seem not to be regulated by their precursor uptake. As shown in Fig. 1, progesterone rather stimulates the uptake of amino acid while it suppresses the incorporation of the amino acid by the embryos. Although progesterone suppresses the uptake of nucleotides and at the same time, reduces their incorporation rate, the absolute value of uptake is far greater than that of incorporation (at least 10 to 40x) and thus the decrease of macromolecular syntheses may not be due to the lack of their precursors. Daniel and Levy (1964) reported that the labelled progesterone in the medium intensely attached to the surface membrane of the embryo or zona pellucida. The present results, however, indicate that progesterone in the medium penetrates into the oocytes or embryonic cells through the surface membrane readily. Moreover, it is generally accepted that quite a number of steroid hormones are able to pass easily through the membrane which contains lipoprotein, since they are nonionic and lipidic in nature (Baulieu, 1975; Gorski and Gannon, 1976).

Therefore, it can be assumed that progesterone in the medium would penetrate into the embryonic cells and directly inhibits the syntheses of macromolecules, which are essential for the embryoaic development. It has been well known that the administration of inhibitors of protein or RNA synthesis such as puromycin or actinomycin D to the embryos at any stage inhibits cleavage and further development of the embryos (Thompson and Biggers, 1966; Molinaro et al., 1972; Golbus et al., 1973). Therefore there is no question about that embryonic protein or RNA synthesis is required for the development. Furthermore, the qualitative pattern of these macromolecules synthesized by the embryos was known to change during early development (Piko, 1970; Knowland and Graham, 1972; McGaughey and Van Blerkom, 1977; Van Blerkom and McGaughey, 1978). Thus, some specific proteins or RNAs would be essential for the

development of early embryos.

From the finding that the incorporation of ^{3}H-uridine into the embryos was most severely affected by the hormone, it is presumed that the RNA may be suppressed at first, followed by a decrease of protein synthesis and DNA synthesis would finally be inhibited by progesterone. Several investigators have studied the effects of estrogen on the metabolism of early mammalian embryos *in vitro*. Their results, however, are conflicting one another. For instance, some investigators suggested that the hormone stimulates the synthesis of protein (Smith and Smith, 1971), and that of nucleic acid (Harrer and Lee, 1973), while others insisted that the hormone neither stimulates nor inhibits the macromolecular synthesis of the embryos (Weitlauf and Greenwald, 1968; Roblero and Izquierdo, 1976; Warner and Tollefson, 1977). These contradictory results seem to be due to the differences in culture methods they employed. Most investigators determined the effects of the hormone by employing Brinster's paraffin drop method. As described previously (Cho et al., 1974), this culture method may not be appropriate for the study on the effects of steroid hormone, because the hormone diffused out into the oil. Considering the results of the present experiments that progesterone have no influence on the metabolism of the embryos at low doses but affect severely at high doses, it is presumed that estrogen may have the same tendency to the metabolism of embryos *in vitro*, that is, the steroid hormone may have no influence on the synthetic activities of the embryos at physiological doses *in vitro*.

The concentration of progesterone in the present study was considerably higher than those contained in follicular fluid of various mammals (Edwards, 1974; Chang et al., 1976) of uterine fluid (Fowler et al., 1977; Borland et al., 1977; Fujimoto and Sundaram, 1978). For example, the peak concentration of progesterone in rabbit uterine lumen at the time of implantation was estimated to about 0.1 μg/ml (Fowler et al., 1977) and that of the follicular fluid ranges from 0.1 to 3 μg/ml in human (Edwards 1974), several μg/ml in monkey (Channing and Coudert, 1976) and 0.2 μg/ml in rabbit (Chang et al., 1976). However, the information concerning the relative proportions of the steroids which are in free and in protein bound form *in vivo* is not available. Since the culture medium of the present study contained none of the follicular or uterine fluid proteins, the free progesterone in the present study is likely to be less effective than that of *in vivo* state. Although caution must be exercised in the interpretation of data from any *in vitro* system, some parallels to physiological processes may be apparent from the data of the present study.

Even though the data obtained here indicated that suppression of macromolecular syntheses may be the cause of the cleavage inhibition of progesterone,

they gave no information on the kind or character of the molecules which are suppressed sensitively. Therefore, further study should be followed in order to identify those molecules in qualitative terms and to clarify their relationship to the hormone action in mammalian embryogenesis.

SUMMARY

Metabolic changes of early mouse embryos treated with progesterone were investigated in order to elucidate the mode of action of progesterone on embryogenesis *in vitro*.

The embryos were cultured, and labelled with radioactive precursors of macromolecules for certain periods in the absence or presence of various concentrations of progesterone by employing the microtube culture technique. The changes of transport and macromolecular synthesis systems of the embryos were examined by measuring the amounts of uptake and incorporation of the precursors.

The results obtained were as follows:

1. Progesterone stimulated markedly the uptake of amino acids, but rather suppressed their incorporation by embryonic cells.
2. Progesterone suppressed both the uptake and incorporation of nucleotide precursors (uridine and thymidine) by embryonic cells.
3. Progesterone penetrated into the embryonic cell membranes and was taken up by them.

The present results seem to indicate that the inhibition of the progesterone on the mammalian embryogenesis *in vitro* may not be directly related to the membrane transport system. They seem to imply that progesterone would penetrate into the embryonic cells and may directly block the biosynehetic pathways of macromolecules, and so lead to the inhibition of the embryogenesis *in vitro*.

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