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Studies on the Maturation of Rabbit Follicular Oocytes *in Vitro* —Effects of Amino Acids and Carbohydrates—

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培養한 토끼 濾胞卵子의 成熟에 관한 硏究 —아미노酸과 炭水化物의 影響에 대하여—

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적 요

토끼 여포난자의 성숙을 유도하는데 필요한 배양액의 성분중 아미노산 및 탄수화물의 영향을 규명하기 위해 실험한 결과는 다음과 같다.

1. 기본배양액에 포함된 탄수화물의 pyruvate, lactate 및 glucose는 모두 난자의 성숙유도에 유효한 성분이었으나 필수영양물질은 아님이 밝혀졌다. 아미노산중에 glutamine과 proline은 난자의 성숙을 촉진하였다. 특히 glutamine은 위의 세가지 탄수화물의 전부 또는 그 각각이 포함된 기본배양액에서 보다 높은 난자의 성숙율을 보였다.
2. 아미노산이 포함된 배양약에 난자를 24시간 배양할 경우 배양 과정중에 생성된 암모니아의 양은 glutamine이 포함된 배양액에서 가장 높았다 (15.2 *ug/ml*). 그러나 이 양은 난자의 성숙을 억제하지는 않았다.
3. 난자의 성숙율은 배양액의 osmole이 270 *mOsm* 일때 가장 높았으나 최적범위는 250~310 *mOsm*로 넓은폭을 보였다.
4. 토끼여포난자는 0.08~2 *m*M의 glutamine과 소혈청단백(BSA)만이 포함된 기본 배양액에서 능히 성숙이 유도됨을 보았다.
5. ^{14}C-glutamine을 사용한 실험에서 glutamine이 토끼난자의 단백질합성과 에너지 공급원으로 이용된다는 사실이 입증되었다.

INTRODUCTION

The metabolic requirements for meiotic resumption in mammalian occytes *in vitro* have been extensively studied but the mechanism involved in the spontaneous maturation has not been fully understood.

Chang (1955) first noted that rabbit serum added to Ringer's solution permitted rabbit follicular oocytes cultured *in vitro* to develop to metaphase II. Biggers *et al.*

(1967) reported that mouse occytes reached metaphase II in chemically defined medium containing pyruvate and oxalcacetate. Similar results were obtained by Zeilmaker *et al.* (1972) with defined medium containing lactate under aerobic conditions if nicotinamide adenine dinucleotide (NAD) is added to the medium. Gwatkin and Haidri (1973) found that hamster occytes underwent maturation in a carbohydrate-containing salt solution supplemented with several amino acids. It is, therefore, assumed that amino acids as well as carbohydrates may serve as energy sources for the maturation of cultured mammalian ooytes.

With amino acids in the medium ammonia formation is expected. Ammonia toxicity for living cells has been well recognized *in vitro* and *in vivo* (Clifford *et al.*, 1969; Prior and Visek, 1972; Visek *et al.*, 1972). There has been no attempt to study the toxicity of ammonia for the mammalian eggs and embryos, although many workers have used various amino acids in culture medium (Kane, 1972; Gwatkin and Haidri, 1973).

The osmolarity of defined medium has been known as an important factor for the maturation of mammalian occytes *in viro.* Biggers *et al.* (1967) found that the optimal osmolarity of defined medium for mouse oocyte maturation was 308 *mOsm.* Gwatkin and Haidri (1973), however, reported that the optimal osmolarity for hamster oocytes was found to be 285~295 *mOsm.* The diversity of these studies has not been explained.

It has been reported that several amino acids stimulate the meiotic resumption in mammalian occytes (Gwatkin and Haidri, 1973; Chung *et al.*, 1974; Bae and Foote, 1975). The mechanism by which glutamine promotes the maturation of mammalian oocytes *in vitro* has not been clarified.

Several investigators have found that protein synthesis takes place during oocyte maturation prior to ovulation, and that RNA and protein synthetic inhibitors and dbcAMP may block ovum maturation (Donhahue, 1968; Stern *et al.*, 1972; Tsafriri *et al.*, 1973; Cho *et al.*, 1974). So far no one has yet attempted to study the meiotic resumption and energy metabolism in rabbit follicular oocytes using chemically defined medium. The rabbit has been known as a good model for these investigations because, as an induced ovulator, accurately timed events *in vivo* can be compared with studies *in vitro.*

The present studies, therefore, were undertaken to elucidate carbohydrate and amino acid requirements, together with the finding of optimal osmolarity and ammonia production, and to determine possible utilization of glutamine for energy and protein synthesis in cultured rabbit follicular oocytes.

MATERIALS AND METHODS

All rabbits used in the present experiments were virgin Dutch belted aged

between 4-7 months old. Ovaries were removed, washed 3 times and transferred to a culture dish containing basic medium under a 5 *ml* layer of mineral oil (viscosity 125/135, Fisher). Follicles ranging from 1 *mm* in diameter up to preovulatory graffian follicles were ruptured with a sharp needle under a stereomicroscope. Oocytes with cumulus cells were sucked into a finely drawn pipette and transferred to a round bottom dish containing medium under the oil layer. By repeated suction and explusion of the oocytes the mucous layer was removed. Oocytes were washed another 3 times in the medium with 0.1% BSA and pooled until incubation. The medium used for washing and handling oocytes before incubation did not contain any carbohydrates or amino acids.

The medium used for this investigation was prepared as follows; all components of the medium (Table 1), except $NaHCO_3$ and bovine serum albumin(BSA, Sigma), were dissolved in redistilled water. Just before use, $NaHCO_3$, carbohydrates, amino acids, as desired, and BSA were added to the medium in that order. The medium was sterilized by filteration with millipore filter. The pH was maintained during storage between 7.1~7.3.

Table 1. Composition of the basic medium (SECM)* and the amino acids used. *(SECM: standard egg culture medium developed by Biggers *et al.*, 1971).

Components	Concentration	
	g/l	*m*M
Basic medium		
NaCl	5.54~6.96	94.7~119.10[a]
KCl	0.356	4.78
Ca-lactate $5H_2O$[b]	0.527	1.71
$CaCl_2$ $2H_2O$	0.256	1.71
KH_2PO_4	0.162	1.19
$MgSO_4$ $7H_2O$	0.293	1.19
$NaHCO_3$	2.106	25.07
Antibiotics: Penicillin G	100 I.U./*ml*	
Streptomycin sulfate	50μg/*ml*	
Bovine serum albumin	4.000	0.4%
Na-pyruvate	0.028	0.25
Na-lactate(60% syrup)	3.68 *ml*	21.55
Glucose	1.000	5.56
Amino acids, when added[c]		
L-glutamine	1.460	10.00
L-methionine	0.447	3.00
L-phenylalnine	0.049	0.30
L-proline	0.115	1.00

a : NaCl was varied to maintain the medium at an osmotic concentration of 308 *mOsm*.
b : Calcium lactate was substituted for $CaCl_2$ in the medium omitted all sources of lactate.
c : When all 4 amino acids were used, each was added at 1/4 the level shown.

The follicular oocytes were then incubated in the culture medium under a mineral oil layer for 18~24 hours at 37°C in 5% CO_2 and 95% air in fully humidifed incubator. After incubation all oocytes were sucked into a finely drawn pipette and washed 3 times in the medium without BSA. Recovered occytes were fixed, stained with 0.25% acetic lacmoid and examined with a phase contrast microscope.

Ammonia concentrations were monitored in media as prepared and after used for oocyte culture as described by Chaney and Marbach (1962). Blanks were run to correct for any ammonia contamination originating in the assay.

The optimal osmolarity of the medium for rabbit oocyte maturation was determined in the medium with a range of 230~350 *mOsm*, which were adjusted by concentrated sodium chloride. The osmols of each medium were adjusted and controlled within the desired range of ±5 *mOsm* (Table 8).

For the determination of possible utilization of glutamine as energy source (Table 10), oocytes from the pooled were washed 3 times with the medium containing 0.1% BSA prior to incubation. For removing cumulus cells, oocytes were transferred to the 10 *ml* test tube containing medium without BSA. They were vortexed for 3 minutes and/or shaken violently by hand. All cumulus cells and corona cells removed were collected by centrifuging at 700 rpm and oocytes were washed 3 times. Collected cumulus cells were not washed again in the medium to minimize losses in handling.

Oocytes and/or cumulus cells were transferred in 2 μl of medium to a tube containing radioactive medium, and this plus a 1,5*ml* ampule for CO_2 absorbant, were placed in a scintillation counting vial. The radioacive medium was prepared as follows; ^{14}C-glutamine (spec. act., 252 *mCi*/*m*M; New England Nuclear) was diluted with 0.4 *m*M cold glutamine (Table 6) to give a final concentration of 0.4029 *m*M. The concentration of ^{14}C-glutamine in the incubation medium was 3 $\mu Ci/ml$.

After incubation for 18 hours at 37°C the CO_2 was driven from the medium by acidifying with 0.1 *ml* of 1N sulfuric acid. One *ml* hyamine hydroxide was added to absorb the CO_2, and the stoppered vials were kept at room temperature for 24 hours. The system for trapping $^{14}CO_2$ was an adaptation of that used by Brinster (1967). Then 14 *ml* of toluene-based scintillation fluid was added, and the radioactivity was determined using a scintillation counter (Nuclear-Chicago, Mark II). Efficiency of carbon dioxide trapping was determined to be 83% when using ^{14}C-bicarbonate.

Protein synthesis was studied in oocytes and cumulus cells incubated for 18 hours at 37°C in a humidified incubator with continuoulsy flowing 5% CO_2 in air (Table 11). Then oocytes were carefully washed 3 times in 0.5 *ml* of the medium

without BSA, and half were transferred to 2 *ml* of the same medium to remove cumulus cells from the oocytes by mixing and shaking.

The other half of the oocytes were separated from the cumulus cells, and the denuded oocytes plus cumulus cells were transferred to a tube containing 0.3 *ml* of the medium without BSA. To break down all cells for protein analysis, repeated freezing and thawing was applied rapidly (Tasca andl Hillman, 1970) by moving the cuture tube between liquid nitrogen and a water bath at 40°C for 25~30 minutes.

Proteins were precipitated by adding 2 *ml* of cold (4°C) 10% trichloroacetic acid (TCA), mixed by a Vortex for 15 seconds and allowed to stand for 15 minutes at 4°C. The precipitate, containing proteins and nucleic acids, was collected on filter (Gelman, type E, fiber glass filter) and rinsed with 10 *ml* of hot (80°~90°C) 5% TCA to remove nucleic acids and tRNA-bound ^{14}C-glutamine (El-Banna and Daniel, 1972). The remaining protein was rinsed with 5 *ml* of cold (4°C) 70% ethanol to facilitate drying. Appropriate blanks were employed to determine background and the efficiency of counting in the scintillation fluid was also measured.

RESULTS

The effects of some carbohydrates on the maturation of rabbit follicular oocytes are shown in Table 2. Development to prophase and metaphase II was similar with each energy source, i.e., pyruvate, lactate and glucose, but the combination was less effective. In the absence of any carbohydrate most oocytes failed to develop past prophase I or degenerated.

Table 2. Effect of some carbohydrates on maturation of oocytes.

Media	No of oocyte cultured	Meiosis I (Met. I)	Meiotic phase of development (% ±S.E.[a])			
			Meiosis II			Degenerate and others
			Prophase	Metaphase	Total of Pro.+Met.[c]	
1. Basic medium	45[b]	37.7±7.2	6.6	13.3	19.9±6.0	44.4±7.4
2. +Pyruvate(P)	47	21.2±6.0	10.6	55.3	65.9±6.9	12.7±4.9
3. +Lactate(L)	48	31.3±6.7	16.7	45.8	62.5±7.0	6.3±3.5
4. +Glucose(G)	45	22.2±6.2	13.3	55.6	68.9+6.9	11.1±4.7
5. +P+L+G	46	32.6±6.9	17.4	39.1	56.5±7.3	10.9±4.6

a Percentages of oocytes reaching at each stage of development afters 18 hours of incubation have been partially combined for simplicity of presentation.

b Seven replicates were done in this experiment with 21 animals.

c Means with different superscripts are different, $P<0.05$.

In the next experiment amino acids were added to the medium (Table 3). The amino acids chosen were based on promising results of previous studies with rabbit embryos (Brinster, 1970) and rabbit oocytes (Chung *et al.*, 1974). Development rates were below normal in this experiment, but both proline and glutamine added separately promoted oocyte maturation. These individual amino acids were as satisfactory as the combination of methionine, phenylalanine, proline and glutamine. Hyaluronidase may have been inhibitory to development. It was not effective in removing the cumulus cells.

Table 3. Effect of amino acid supplementation on oocyte development[a].

Media	No of oocytes cultured	Developed to prophase or metaphase II(%±S.E.)
Basic medium plus three carbohydrates[b]		
Control	29	41±9.1
+Glutamine	35	54±8.4
+Methionine	35	38±8.2
+Phenylalanine	34	35±8.2
+Proline	35	57±8.4
+All 4 amino acids	38	55±8.1
+All 4 amino acids+hyaluronidase treat.[c]	39	44±8.0

a Incubated for 24 hours.
b For composition see Table 1.
c Hyaluronidase treatment; the oocytes were treated with 300 USP units/*ml* for 30 minutes before culture.

Levels of ammonia in the culture media are shown in Table 4. The highest level of ammonia was in the medium containing glutamine both before and after culture. The initial level is indicative of spontaneous degradation of the amino acid. Increased ammonia produced during culture could result from metabolism by the follicular cells and/or the oocyte of the amino acids and possibly of BSA. However, ammonia production was minimal and did not produce any detectable effect on maturation.

Table 4. Concentration of ammonia in various media before and after culture.

Media	Ammonia level, $\mu g/ml$±S.E.	
	Before culture	After 24 hours of culture
Basic medium[a]	1.38±1.34	4.5. ±2.76
+Glutamine	10.20±0.27	15.22±0.88
+Methionine	−1.38±0.50[b]	1.38±1.00
+Phenylalanine	−0.35±0.25[b]	1.12±0.35
+Proline	0.86±0.38	0.26±0.87
+All 4 amino acids	3.29±0.63	8.04±0.71
+All 4 amino acids+hyaluronidase treat.	2.59±0.38	6.57±0.36

a For composition see Table 1. All media contained 0.4% BSA unless otherwise indicated. When 4 amino acids were combined each was used at 1/4 of the individual concentration.
b Negative values were below the blanks and are presumed to equal zero.

Because of the beneficial effects of glutamine (Table 3) and the report by Kane (1972) that rabbit zygotes could develop without a simple carbohydrate source of energy in the medium an experiment was designed to compare the medium with or without carbohydrates and glutamine. Results are summarized in Table 5. A highly significant improvement ($P<0.005$) in oocyte maturation was associated with glutamime in the medium. The effect of glutamine appeared to exceed that of the carbohydrate combination of glucose, pyruvate and lactate. These carbohydrates exerted no beneficial effect when added to the medium containing glutamine.

Table 5. Effect of carbohydrates and glutamine on oocyte development.

Basic medium			
Carbohydrates (pyruvate, lactate and glucose)	Glutamine	No of oocytes cultured	Developed to prophase or metaphase II in 24 hours (% ±S.E.)
−	−	55	35±6.4
+	−	53	49±6.9
−	+	53	70±6.3
+	+	52	67±6.5

Because of the highly beneficial effect of glutamine various concentrations of this amino acid were tested (Table 7). From the table it can be seen that without glutamine in this carbohydrate-free medium 55% of the oocytes did not develop beyond prophase I. Oocyte development in the optimum levels of 0.08, 0.4 and 2.0 *m*M of glutamine was higher ($P<0.01$) than when 0 or 50 *m*M of glutamine

Table 6. Composition of the medium used for the effect of different concentrations of glutamine.

Components	Concentration	
	g/l	*m*M
NaCl.	6.960	119.07
KCl	0.356	4.78
$CaCl_2$ $2H_2O$	0.256	1.71
KH_2PO_4	0.162	1.19
$MgSO_4$	0.293	1.19
$NaHCO_3$	2.106	25.07
Antibiotics: Penicillin G	100I.U./*ml*	
Streptomycin sulfate	50*μg/ml*	
Bovine serum albumin	4.0(0.4%)[a]	—
	1.0(0.1%)	—
L-Glutamine[b]	0.0117~7.3	0.08~50

a : For incubation, 0.4% BSA was employed.
b : Glutamine concentration of each medium used is shown in Table 7.

were added. This high development rate in the presence of glutamine is in close agreement with the previous experiment (Table 5) in which 70% of the oocytes were found to develop with 10 *m*M of glutamine added to a similar medium. Thus the upper limit of the optimum level of glutamine appears to approach 10 *m*M.

Table 7. Effects of different concentration of glutamine on maturation of the cocytes *in vitro*.

Medium	No. of oocytes cultured	Meiotic phase of development, %[a]					
		Meiosis I			Meiosis II		Degenerate and others
		D.	P.	M.	P.	M.	
Basic medim							
0.0 *m*M glutamine	40	20.0	35.0	2.5	25.0	5.0	12.5
0.08*m*M glutamine	37	16.2	2.7	5.4	48.6	24.3	2.7
0.04*m*M glutamine	36	11.1	2.8	5.5	13.9	55.6	11.1
2 *m*M glutamine	38	10.5	—	13.2	15.8	55.3	5.3
10 *m*M glutamine	39	17.9	5.1	15.4	17.9	41.0	2.6
50 *m*M glutamine	53	9.4	11.3	1.9	11.3	34.0	32.1

a : Percentage of oocytes reaching at each stage of development after 18 hours of incubation. No oocytes were found in anaphase I or telophase I.

The effect of osmolarity in the culture medium (Table 8) for the maturation of rabbit follicular oocytes are shown in Table 9. The optimal osmolarity was found to be 270 *mOsm* with a wide range from 250 to 310 *mOsm*. As shown in the table much percentage of oocytes remained at earlier stages of maturation at either higher or lower levels of osmolarity. This indicates that oocytes could not progress to the developed phases such as prophase II and metapase II at these extreme osmolarity.

Table 8. Composition of the medium used for the effect of osmolarity on rabbit follicular oocytes.

Component	Concentration	
	g/l	*m*M
NaCl	4.6287~8.1351	79.357~139.209
KCl	0.356	4.78
$CaCl_2.2H_2O$	0.252	1.71
KH_2PO_4	0.162	1.19
$MgSO_4$ $7H_2O$	0.294	1.19
$NaHCO_3$	2.106	25.07
Glutamine	0.292	2.0
Bovine serum albumin	0.4%	—
Antibiotics: Penicillin G	100I.U./*ml*	—
Streptomycin sulfate	50*μg/ml*	—

Table 9. Nuclear phases of the cultured oocytes in various osmols.

Replicate	Medium[a] with glutamine+0.4% BSA	Meiotic phase[b]								
		D	PI	MI	AI	TI	PII[c]	MII	Other Degen.	Total
1.	230 *msOm*	13	9	6			2	16	3	49
2.	250 〃	7	2	6		1	2	33	4	55
3.	270 〃	9	4	3			9	27	1	53
4.	290 〃	8		4			5	30	8	53
5.	310 〃	9	1				8	23	7	48
6.	330 〃	6	8	5			2	22	8	51
7.	350 〃	16	11	4	1		2	6	12	52

a See the text for the preparation.
b D, dictyate; PI, lst prophase; MI, lst metaphase; AI, lst anaphase; TI, lst telophase; PII, 2nd prophase; MII, 2nd metaphase; Other, non-identified nuclear phase; Deg., degenerating oocyte.
c PII, the oocyte which formed lst polar body, but whose chromosomes remained still in prophase arrangements.
Seven replicate, 25 animals.

Table 10 represents the results of glutamine utilization by rabbit occytes for their energy source. It is very difficult to separate the cumulus cells from follicular oocytes in the rabbit as they have attachments through the zona pellucida (Zamboni and Mastroianni, 1966). Vortex mixing for 5 minutes in a preliminary experiment damaged the oocytes, as judged by the fact that only 20% of the oocytes so treated were able to develop to prophase II or metaphase II. Consequently, with the mild Vortex treatment for 3 minutes used in the present study (Table 10) some cumulus cells were attached to the oocytes. This fact may explain part of the variability among replicates in CO_2 production per oocyte in the 'oocyte only' treatment. However, it is clear that many cumulus cells were

Table 10. Oxidation of ^{14}C-glutamine in rabbit oocytes and cumulus cells cultured for 18 hours.

Relicate	$^{14}CO_2$, cpm				
	Oocytes only (A)	Cumulus cell only (B)	(A+B)	Oocytes-cumulus cells (incubated after separation) (C)	Oocyte-cumulus cells (incubated intact) (D)
1.	350(26)	943	1293	1073(27)	1659(30)
2[a].	443(17)	2492	2935	1720(16)	2268(16)
3.	205(19)	933	1138	898(19)	1380(17)
4[a].	594(16)	1219	1813	2007(16)	1285(14)
Mean	422	1306	1728	1416	1513
±S.E.	±68	±301	±323	±204	±218

Numbers of oocytes are shown in parentheses.
a In these replicates some cumulus cells remained on most oocytes.

freed, based upon microscopic observations and the fact that the net CO_2 cpm per cumulus cells group was approximately 3 times the cpm average per 'oocyte only' group. Variability in counts among the cumulus cells could also result partly from losses during processing and from unequal numbers of cumulus cells and stages of oocytes among the replicates.

It is noteworthy that the amount of $^{14}CO_2$ released by groups A+B, C and D (Table 10) was similar ($P<0.10$). The fact that utilization of gltutamine proceeded similarly whether or not the cumulus cells and oocytes were in intimated structural contact or even in the same cultue tube, is interpreted to mean that the oocytes were not dependent upon some metabolic transformation by the cumulus cells or *vice versa*. Futhermore, it appears that the physical treatment imposed to separate the components did not damage the systems utilizing glutamine. Clearly the cumulus cells per occyte utilized about 3 times as much glutamine as did oocytes, but on a per cell basis the oocytes utilized much more glutamine. The present studies do not permit a comparison to be made on the basis of total mass because the hydrolysis to release CO_2 degraded the cells, preventing further chemical analysis.

Table 11. Incorporation of universally labeled ^{14}C-glutamine into TCA precipitable material produced by rabbit oocytes and cumulus cells during culture for 18 hours.

Replicate	Oocyte only (cpm)	Oocyte+cumulus cell (cpm)	Ratio between groups
1.	30(28)	61(30)	2.0
2.	24(22)	37(20)	1.6
3.	17(18)	30(19)	1.7
4.	27(20)	44(21)	1.6
5.	18(26)	31(26)	1.7
Mean±S.E.	23.2±2.52	40.6±5.68	1.72±0.07

Numbers of oocytes are shown in parentheses.

Incorporation of ^{14}C-glutamine into the protein (10% TCA-precipitable material) of oocytes and cumulus cells is peseented in Table 11. The ratio in average cpm between 'occytes only' and oocytes plus cumulus cells of 1.72 indicates that the cumulus cells were not synthesizing as much protein as the oocyte. From the mean values in Table 11, and assuming no contamination of the oocytes with cumulus cells, it would seem that the cumulus cells per oocyte synthesized about 75% as much protein as did the oocytes. This is in marked contrast to the ratio of glutamine utilized for energy by the two types of cells.

DISCUSSION

It has been shown that some carbohydrates are utilized as an energy source for the meiotic resumption in cultured mammalian oocytes (Biggers *et al.*, 1967; Donhahue, 1968; Zeilmaker *et al.*, 1972; Gwatkin and Haidri, 1973). Donahue (1968) suggested that pyruvate was the most important carbohydrate for the maturation of mouse oocytes. Biggers *et al.* (1971) reported that pyruvate, oxaloacetate and to some extent lactate, but not glucose or phosphoenolpyruvate, can be served as energy sources for the mouse oocyte maturation. The present results, however, showed that pyruvate, lactate and glucose are all independently effective for the meiotic resumption in rabbit follicular oocytes. Different requirements between these two species may be due to the different culture system.

For amino acid requirements, Gwatkin and Haidri (1973) were first to report that each of four amino acids, i.e., isoleucine, glutamine, phenylalanine and methionine equally promoted the maturation of hamster oocytes in the presence of carbohydrates in the medium. The present results showed that glutamine and proline were more effective than others in inducing the maturation of rabbit oocytes *in vitro*. The diversity of these results on two species might be caused by different culture system and by species-specificity. It was also found that various concentrations of these carbohydrates and amino acids promote development of mammalian embryos depending in part upon the species and stage of development. (Brinster, 1965, 1970, 1971; Biggers *et al.*, 1967; Cross and Brinster, 1970; Kane, 1972).

The ammonia production during the culture of mammalian oocytes *in vitro* has not been previously reported. The ammonia level produced in the present studies did not show any detectable effect on the maturation of rabbit follicular oocytes (Table 5). The present results are consistent with those of mammalian somatic cells *in vitro* (Visek *et al.*, 1972).

It was reported that rabbit zygotes could develop in a medium containing a variety of amino acids, and that the inclusion of simple carbohydrates as energy substrates had no effect (Kane, 1972). Gwatkin and Haidri (unpublished) found that hamster oocytes can mature in the medium containing BSA or 12 amino acids but devoid of carbohydrates. These results may suggest that amino acids alone can be served as a sole energy souce for the development of mammalian oocytes and embryos *in vitro*. The present studies clearly demonstrated that the presence of glutamine in the medium effectively promoted the maturation of rabbit oocytes.

Follicular cells are known to be able to metabolize some carbohydrates and supply oocyte with products which permit maturation in the mouse (Biggers *et*

al., 1967; Donahue and Stern, 1968; Cross and Brinster, 1970). Removal of the follicular cells in the rabbit, cow and human reduced the number of oocytes maturing *in vitro* (Kennedy and Donahue, 1969; Robertson and Baker, 1969). The present results with rabbit oocytes attached with cumulus cells are interpreted to indicate that glutamine supplied a useful component to the oocytes, which in its absence was not supplied by the medium directly or by metabolic reactions of the cumulus cells.

The present studies showed that some oocytes did develop to metaphase II in the control medium without carbohydrates and amino acids (5-13% in Table 2 and 7). This result may suggest that BSA is beneficial to oocytes as does to embryos (Glass, 1963; Brinster, 1965). Oocyte development is poor in the basic salt medium devoid completely of carbohydrates, amino acids and BSA (Chung *et al.*, 1974). These studies clearly indicate that merely pricking the follicle does not lead to extensive meiotic development of rabbit oocytes out of the body. However, the stage of follicular development may also affects the oocyte maturation. In fact, the amino acid requirement in the hamster increases as ovulation approaches (Haidri and Gwatkin, 1973). Poor oocyte development at the higher concentrations of glutamine seems to be ceaused by higher osmolarity due to the addition of glutamine in the medium.

One of the possible explainations for the difference between the requirement of rabbit oocyte and that of oocytes from spontaneous ovulators is that the rabbit oocyte may accumulate nutrients and store them readiness for induced ovulation. This could be in contrast to cyclical ovulators in which programmed cyclical changes in oocyte nutrition prior to the initiation of ovulation might occur. However, direct comparisions between the nutrient accumlation of rabbit oocyte and the metabolism of oocytes in spontaneous ovulators have not been reported.

The osmolarity of defined media for the maturation of mammalian oocytes has been reported in mouse and hamster oocytes (Biggers *et al.*, 1967; Gwatkin, 1972; Gwatkin and Haidri, 1973). The present result in rabbit oocytes showed a considerably wide range of optimal osmolarity comparing to the previous reports. This may be caused by the cumulus cells which may act as a buffer on drastic changes of the medium environment.

It has been suggested that certain amino acids and possibly proteins may be utilized as energy sources by the young embroyos (Brinster, 1970). Kane (1972) reported that the rabbit zygote could develop into blastocysts in defined medium with appropriate amino acids but devoid of carbohydrates. The present study showed a surprising finding that development was equally successful in the glutamine-containing medium when all carbohydrates were ommited. Thus, it was conclusively proved that glutamine provides a source of energy. Cross (1973) has

found that cumulus cells do not improve oocyte maturation or fertilization *in vitro* in the mouse, although they may play a post-ovulatory role in fertilization in the hamster (Gwatkin *et al.*, 1972). The utilization of glutamine by the cumulus cells for energy in this study was about three times that of the associated oocyte. While the relative mass is unknown, it appears that the oocyte may either have a lower energy requirement than the cumulus cells or use in part an endogenous source of energy. The metabolic pathway of glutamine utilized as energy source remains to be elucidated.

Utilization of amino acids by oocytes and young embryos for protein synthesis has been widely investigated (Mintz, 1964; Monsei and Salfi, 1967; Tasca and Hillman, 1970; Stern *et al.*, 1972). Stern *et al.* (1972) concluded that protein synthesis *de novo* was necessary for normal maturation of the mouse oocyte. The present studies showed that more glutamine was incorporated into protein by the oocyte than by the cumulus cells. In contrast to the utilization of glutamine for energy by the two components this finding clearly indicates that protein synthesis is occurring much more in the oocyte associated with the phases of meiosis than in the cumulus cells. In view of these results showing that oocyte-development is improved by the addition of glutamine to a salt carbohydrate-BSA medium and that glutamine can replace the carbohydrates in such a medium, a central role of glutamine for the development of rabbit follicular oocyte *in vitro* is indicated. Presumably it may play a vital role relative to protein synthesis *in vivo* also where other sources of energy would be reaily available.

SUMMARY

Rabbit follicular oocytes were cultured in a basic medium containing 0.4% bovine serum albumin (BSA), carbohydrates and amino acids in various combinations. Osmolarity of the medium was maintained at 308 *mOsm*.

The carbohydrates, pyruvate, lactate and glucose were all about equally beneficial, but not essential for rabbit oocyte maturation. Glutamine and proline, but not methionine or phenylalanine stimulated oocyte develoment. Glutamine stimulated more follicular oocytes to develop to prophase and metaphase II than did any of the three carbohydrates tested alone or in combination.

Ammonia production after 24 hours of culture was highest in medium containing glutamine(15.2 $\mu g/ml$) but this was not inhibitory to maturation. Negligible amounts of ammonia were found with the other amino acids added.

The optimum level of osmolarity for rabbit oocyte maturation appears to be ranged from 250~310 *mOsm* with the maximum level of 270 *mOsm*.

With 0, 0.08, 0.4, 2, 10 and 50 *mM* of glutamine in the medium, plus BSA

but without carbohydrates, 30, 73, 70, 71, 59, 45% of the oocytes developed to prophase or metaphase II respectively. This indicates that no carbohydrate is required for the maturation of rabbit oocytes when 0.08~2 *m*M of glutamine is included, which are the optimum range.

Follicular oocytes could develop in the medium containing ^{14}C-glutamine and BSA but without carbohydrates or other organic compound. From the $^{14}CO_2$ produced and TCA precipitable material isolated, it is suggested that glutamine probably is utilized by oocytes and cumulus cells as a source of energy as well as for protein synthesis.

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