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Studies on the Effects of Dibutyryl Cyclic AMP and Theophylline on Intracellular Contents of Glycogen of Mouse Follicular Oocytes *in Vitro*

Wan Kyoo Cho and Yong Dal Yoon
(Dept. of Zoology, Seoul National University)

Dibutyryl Cyclic AMP와 Theophylline이 培養중인 생쥐 濾胞卵子의 Glycogen함량에 미치는 영향에 관한 연구

趙 完 圭·尹 用 達
(서울대 自然大 動物學科)

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적 요

dbcAMP와 theophylline이 난자의 성숙을 억제하는 기작과 난자내 glycogen 함량과의 관계를 밝혀보기 위하여 실험한 결과는 다음과 같다.

1. 생쥐 여포난자의 PAS 양성물질은 glycogen이며 핵의 성숙분열이 진행됨에 따라 glycogen의 함량은 감소한다.

2. dbcAMP나 theophylline에 의해 핵의 성숙이 억제된다 하더라도 난자내의 glycogenolysis는 촉진된다. 이에 반해 핵분열이 일어나지 않은 난자는 배양이 진행되더라도 glycogen을 그대로 유지하고 있다. 일단 dbcAMP나 theophylline에 의해 성숙이 억제되었던 난자가 성숙과정에 들어가려면 다시 glyconeogenesis가 일어나 세포질내의 glycogen의 양이 회복하며 이때의 glycogen은 핵성숙과 더불어 소모된다.

3. Glycogen의 회복은 배양액내의 glucose 유무에 관계없이 이루어지며 따라서 이는 난자내 glucose 혹은 다른 전구물질에 의해 이루어지리라고 추측된다.

4. 결국 dbcAMP나 theophylline은 난자내 cAMP의 양을 증가시켜 glycogen의 소모를 일으키는 것으로 생각되며, glycogenolysis와 난자의 핵 성숙과정이 별개로 진행되기는 하지만 성숙의 요건은 일정량 이상의 glycogen이 함유되어 있어야 한다는 것을 알게 되었다.

INTRODUCTION

It has been reported that dibutyryl cyclic AMP (dbcAMP), a derivative of adenosine-3′, 5′ cyclic monophosphate (cAMP) or theophlline, an inhibitor of

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phosphodiesterase activity by which cAMP is converted to 5′-AMP, inhibits maturation of mouse oocytes *in vitro* (Cho *et al.*, 1974). Jost and Rickenberg (1970) and Robison *et al.* (1971) demonstrated that the intracellular level of cAMP was increased after treatment with dbcAMP or theophylline. These findings suggest that the inhibitory action of dbcAMP or theophylline might be caused by an increase of cAMP level in the oocytes. Until today, however, there has been no paper regarding to the role of cAMP but a few of dbcAMP or theophylline on the meiotic resumption of oocytes, in spite of a number of papers reporting the response of tissue cells to the cyclic nucleotides.

Sutherland and Robison (1969) observed the glycogenolysis induced in the liver cells by the treatment with cAMP. It was reported that increased cAMP by dbcAMP or theophylline agents decreased the amount of glycogen in the cell (Hilz and Tarnowski, 1970; Heersche *et al.*, 1971 ; Zieve and Schumkler, 1971 ; Kaukel *et al.*, 1972 ; Opler and Makman, 1972). Ryan and Heidrick (1968), Abell and Monahan (1973) and Erdström *et al.* (1974) stated that the cAMP added to the medium suppressed the mitosis.

Recently, Stern and Wassarman (1974) found that the oocytes in the presence of inhibitor such as dbcAMP underwent the protein synthesis as much as the untreated one even though the treated oocytes were arrested at the dictyate stage. Cho and Yoo (1975), working with the oocytes treated with dbcAMP or theophylline, found that RNA synthesis which normally takes place in 2~6 hours of incubation was completely suppressed, but by removal of the agents the oocytes started to form RNA and at the same time the germinal vesicle began to break down for further meiotic division. Thus, dbcAMP or theophylline directly or indirectly suppresses the synthesis of meiotic RNA which is possibly essential for the formation of specific proteins which act on the breakdown of germinal vesicles of the oocytes.

It is assumed that if dbcAMP or theophylline causes the increase of intracellular level of cAMP, the glycogen in the oocytes treated with either of these agents would be degraded due to the glycogenolysis which is a response of the cell to cAMP.

The present studies, therefore, were planned to examine the glycogenolysis in the oocytes in the presence of dbcAMP or theophylline applying PAS reaction and microspectrophotometry, and to understand the inhibitory mechanism of the agents.

MATERIALS AND METHODS

Three to four week old A-strain female mice bred randomly in our laboratory were used for donor of the oocytes. The ovaries were taken out from the body cavity, and transferred to the Dulbecco's phosphate buffered saline (PBS) to

wash and trim fats and blood clots out from the ovarian surface. The cleaned ovaries were again transferred to a watch glass containing 1 m*l* of the modified Krebs-Ringer bicarbonate solution (Biggers *et al.*, 1971) which was the basic medium throughout the present experiments. Ovarian follicles were punctured by a syringe needle (Gauge #27) in the medium in order to expel the oocytes out. The oocytes were collected in the medium by a capillary pipette connected to a mouth piece, and washed three times with a fresh medium. Only the oocytes denuded from cumulus cells and intact with clear germinal vesicle were used as materials.

Ten mg each of dbcAMP (N^6, $0^{2\prime}$-dibutyryl cyclic AMP, Sigma) or theophylline (Sigma) was dissolved in 1 m*l* of PBS as a stock solution, and they were kept at -20°C until use. At the time of culture, a desired concentration of dbcAMP or theophylline was diluted in the culture medium. Equal volume of plain PBS was added to the agent-free medium to be served as a control medium. The culture period was varied according to the experimental design.

The culture system was mainly adopted by the Brinster's method (1963). Ten-20 dictyate oocytes were introduced by a capillary pipette into a drop of medium (50 μl) set on the bottom of a plastic dish (Falcon #3002) containing 15 m*l* of mineral oil previously equilibrated with 5% CO_2 in air for 2~3 hours. After setting the oocytes in the culture dish they were placed in an incubator at 37°C supplied with 5% CO_2 in fully moistened air, and were left for a desired period. After the incubation the oocytes were taken out from the dish and were processed for the PAS reaction. The PAS reaction was carried out by the method of Thomson and Brinster (1966).

The detailed procedures used were as follows: The oocytes washed several times in 0.154 M saline, and those contained in a very small volume of the saline in a tip of the capillary pipette were placed on a slide glass. Those set on the slide glass were fixed with gas of a primary fixative (formalin 1 : acetic acid 1) in a covered coplin jar for 5~10 min. Thereafter, the occytes were fixed with Tellyesniczky's fixative which is allowed to pass over the oocytes from one end of the slide to the other. The zona pellucida was dissolved within 20 min by the fixative and the eggs adhered firmly on the surface of the slide. After withdrawal of the remaining fixative on the slide glass, it was preserved in 70% ethanol at 4°C for staining. Before staining the oocytes on the slide glass, they were washed thoroughly with distilled water, and oxidized with 0.5% periodic acid for 10~15 min. The slide was then washed again in running water for 10 min and then with fresh 0.5% $Na_2S_2O_5$ three times for 2 min, followed by staining with a cold Schiff's reagent for 15~20 min. After the staining, the oocytes were covered with a cover glass for observation.

These oocytes were then applied for the photomicroscopic determination of glycogen. The intensity of PAS reaction in the oocytes was observed through Wild M 20 microscope.

For microspectrophotometry, the oocytes stained with Schiff's reagent were mounted with Cargile oil (refractive index: 1.561) and covered by a cover glass (Gold Seal, thinness No. 1). Continuous absorption spectra in cytoplasmic points of the oocytes stained with PAS were found at the range of 480-600 nm on the Olympus Model A-4 microspectrophotometer (Fig. 1). The measurement was followed by two wavelength method (Mendelsohn, 1958), using 500 nm and 560 nm wavelengths, to determine glycogen content of the whole ova. The oocytes were observed by 10×10 magnification throughout the spectrophotometry.

All glasswares and equipments used for the culture were sterilized by hot air sterilizer and the media by Millipore filter.

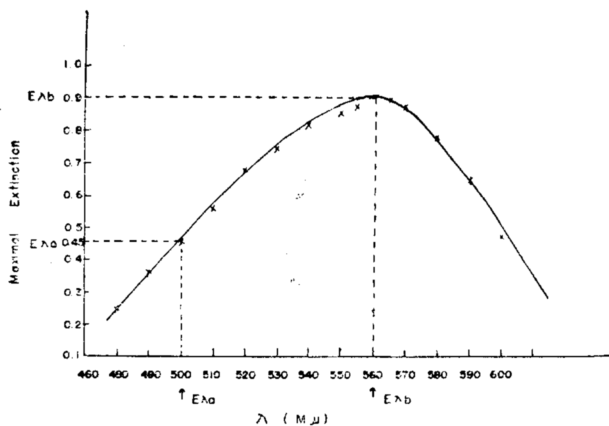

Fig. 1. Absorption spectra of glycogen Feulgen dye (basic fuchsin) complex in mouse oocytes.

RESULTS

Firstly, to confirm that the PAS positive material in the oocytes is glycogen, the oocytes previously treated with amylo-1, 4-α-1, 6-glucosidase were stained with Schiff's reagent, and the intensity of the reaction to PAS in the oocytes was compared with that of non-treated one. The results are shown in Table 1. As shownin the table, it was found that the initial reaction in the oocytes was strong. This means that the oocytes freshly collected from the follicles possess a large amount of glycogen. Secondly, for an aim of the determination of the spectral curve of the extinction of PAS positive materials in the oocytes, those treated with Schiff's reagent were set for a spectrophotometry.

Table 1. PAS reaction of the dictyate oocytes previously treated with amylo-1, 4-α-1, 6-glucosidase for 3 hours. Intensity of the reaction was represented by arbitrary units obtained by microspectrophotometry.

Treatment	No. of oocytes	Arbitrary unit/ovum ±S.D.	S.E.
None	13	2,554±449[1)]	33.8
Amylo-glucosidase in acetate buffer	17	705±143	6.5
Amylo-glucosidase in phosphate buffer	6	500±174	34.8

1) Because of the strong reaction to the PAS treatment, the real units comparable to the intensity was not available. The units shown here is only represented by the units measurable in the scale of the microspectrophotometer. The real one would be far beyond of this units.

All oocytes freshly collected from the follicles showed strong reaction to the PAS. As the meiotic division progressed, the reaction was gradually decreased, and the least figures were obtained in the oocytes at metaphase II (Table 2, Fig. 3A). This implies that the glycogen in the oocytes is consumed during the meiotic resumption. In other words, the degradation of glycogen is necessary for the oocyte maturation.

The oocytes exposed to dbcAMP (100 $\mu g/ml$) or theophylline (100 $\mu g/ml$) for 20 hours in the medium kept their germinal vesicle intact but the PAS reaction was weak as in the oocytes on the regular meiosis (Fig. 3 B, C). This means that the oocytes, even though the meiotic division is suppressed by the agents, consume glycogen well during the cultivation. Such weakening of the PAS reaction was not seen in the oocytes which were unusually kept at the arrested state through culture in the plain medium. That is, two kinds of dictyate oocytes, one induced by the agents and the other induced spontaneously in the untreated medium, show the different response to the PAS. Thus, the ocotyes under the influence of the

Table 2. PAS reaction of the oocytes cultured for 20 hours in the plain medium. Intensity of the reaction was represented by arbitrary units obtained by micro spectrophotometry.

Nuclear stage of oocytes	No. of oocytes	Arbitrary unit/ovum ±S.D.	S.E.
Dictyate[1]	15	2,456±449[2]	116
Metaphase I	20	1,664±192	41
Metaphase II	25	1,481±188	38

1) The oocytes which had been arrested at the dictyate stage throughout the incubation were tested.
2) Same as 1) of Table 1.

Table 3. Comparison of intensities of the PAS reaction between the fresh dictyate oocytes and the oocytes whose meiotic division had been inhibited by dbcAMP(100 $\mu g/ml$) or theophylline (100 $\mu g/ml$) for 20 hours.

Experiments	Medium containing dbcAMP			Medium containing theophylline			fresh oocytes[1]		
	No. of oocytes	intensity strong	intensity weak	No. of oocytes	intensity strong	intensity weak	No. of oocytes	intensity strong	intensity weak
1	6	1	5	5	0	5	11	8	3
2	9	0	9	9	2	7	21	17	4
3	21	2	19	23	4	19	27	23	4
4	15	3	12	13	3	10	17	13	4
Total	51	6	45	50	9	41	76	61	15
%		11.8	88.2		18.0	82.0		80.3	19.7

1) The dictyate oocytes were stained by PAS immediately after liberation from ovarian follicles.

agents stimulate the glycogen breakdown even though nuclear maturation is suppressed, while those with intact germinal vesicles in the plain medium keep the glycogen unchanged throughout the period of incubation. The results of the PAS reaction obtained from the oocytes cultured in the presence of the agents for 20 hours and from the oocytes freshly recovered from the follicles were set in Tables 3 and 4.

Table 4. PAS reaction of the oocytes cultured for 20 hours in the medium containing dbcAMP or theophylline. Intensity of the reaction was represented by arbitrary units obtained by microspectrophotometry.

Treatment	No. of oocytes	Arbitrary unit/ovum ±S.D.	S.E.
None[1]	15	2,456±449[2]	116
dbcAMP 100 μg/ml	10	1,116±111	39
theophylline 100 μg/ml	10	1,311±118	42

1) The oocytes which had been arrested at the dictyate stage throughout the incubation in the plain medium were tested.
2) Same as 1) of Table 1.

An experiment was done to see the effect of glucose in the medium on the glycogenolysis which was induced by dbcAMP or theophylline during culture.

Table 5. Effect of glucose on the PAS reaction of the oocytes cultured for 20 hours in the presence of inhibitors. Intensity of the reaction was shown by arbitrary units.

Concentration of glucose	Medium containing			
	dbcAMP (100 μg/ml)		theophylline (100 μg/ml)	
	No. of oocytes	Arbitrary unit per ovum±S.D.	No. of oocytes	Arbitrary unit per ovum±S.D.
16.65 mM	8	1,298±162	15	1,694±276
5.55 mM	9	1,119±80	20	1,285±296
0 mM	15	994±190	10	1,085±137

Table 5 is the summary of the results obtained from the cultures with 16.65 mM, 5.55 mM or 0 mM of glucose in the medium containing the agents. As shown in the table, the oocytes cultured in the medium with 16.65 mM glucose showed relatively strong reaction to the PAS treatment than those cultured under lower concentrations of glucose.

The oocytes which consumed most of glycogen during the culture in the presence of the agents restored the glycogen within two hours when they were transferred into the plain medium (Fig. 3 D,E), and they started their meiotic resumption even though there was a slight delay (Fig. 2). The intensity of the PAS reaction of such oocytes was strong as the fresh oocytes obtained from the follicle (Tables 6, and 7).

Table 6. PAS reaction of the oocytes transferred and cultivated for 2 hours in the plain medium after culture in the presence of the inhibitors for 20 hours. The intensity of the reaction was shown by arbitrary units.

Nuclear stage of oocytes	Medium containing			
	dbcAMP (100 μg/ml)		theophylline (100 μg/ml)	
	No. of oocytes	Arbitrary unit per ovum±S.D.	No. of oocytes	Arbitrary unit per ovum±S.D.
Dictyate	10	2,338±495[2]	10	2,465±393[2]
GVBD oocytes[1]	10	1,988±205	10	2,234±232

1) The oocytes already started for the meiotic division.
GVBD: Germinal vesicle breakdown.
2) Same as 1) of Table 1.

Table 7. PAS reaction of the oocytes cultured in plain medium for 2 hours after cultivation in the presence of dbcAMP (100 μg/ml) or theophylline (100 μg/ml) for 20 hours.

Experiments	dbcAMP			theophylline		
	No. of oocytes	intensity		No. of oocytes	intensity	
		strong	weak		strong	weak
1	18	7	11	14	4	10
2	14	5	9	21	7	14
3	11	5	6	10	6	4
4	13	5	8	7	5	2
5	10	5	5	6	5	1
Total	66	27	39	58	27	31
%		40.9	59.1		46.6	53.4

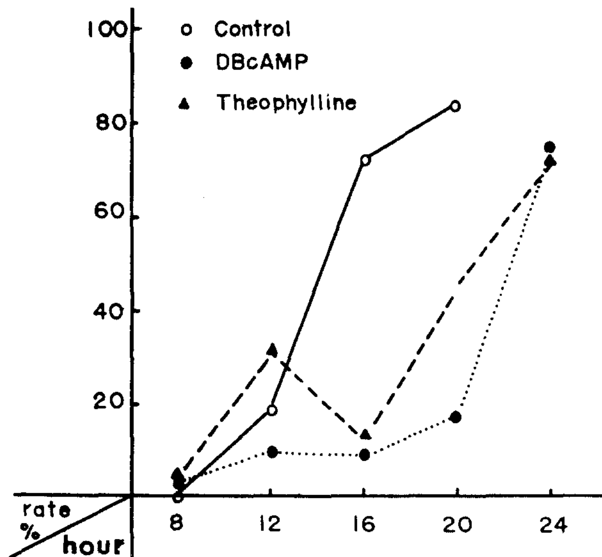

Fig. 2 Nuclear maturation of mouse oocytes in plain medium in 20 hours after exposure to dbcAMP or theophylline for 20 hours *in vitro*.

In order to examine the effect of glucose in the medium on the restoration of glycogen in cultured oocytes, which have shown a weak response to the PAS treatment, the oocytes were transferred to the plain medium containing 16.65 mM or 0 mM of glucose. The results are shown in Tables 8 and 9. It was found that the ablility of the regaining of glycogen by the oocytes was actually not depended on the exogenous glucose.

Table 8. Effect of exogeneous glucose on PAS reaction in the oocytes transferred and cultured for 2 hours in the plain medium after cultivation in the presence of the inhibitors for 20 hours. Intensity of the reaction is shown by arbitrary units.

Concentration of glucose	Medium containing dbcAMP (100 μg/ml)		theophylline (100 μg/ml)	
	No. of oocytes	Arbitrary unit per ovum±S.D.	No. of oocytes	Arbitrary unit per ovum±S.D.
16.65 mM	5	2,407±246[1]	5	2,307±116[1]
0 mM	5	2,761±199[1]	5	2,629±353[1]

1) Same as 1) of Table 1.

Table 9. Effect of exogeneous glucose on PAS reaction in the oocytes cultured for 2 hours in the plain medium after culture in the presence of dbcAMP (100 μg/ml) or theophylline (100 μg/ml) for 20 hours.

Concentration of glucose	Experiments	Medium containing dbcAMP			theophylline		
		No. of oocytes	Intensity		No. of oocytes	Intensity	
			strong	weak		strong	weak
16.65 mM	1	12	6	6	18	10	8
	2	10	8	2	8	6	2
	Total	22	14	8	26	16	10
	%		63.6	36.4		61.5	38.5
0 mM	1	18	8	10	8	6	7
	2	10	8	2	8	6	7
	Total	28	16	12	26	12	14
	%		57.1	42.9		46.2	53.8

The test was done applying mbcAMP (100 μg/ml), and the efficiency of the agent to the inhibition or glycogenolysis was found to be quite similar to that of the dbcAMP.

DISCUSSION

Thomson and Brinster (1966) and McReynold and Hadek (1972) found that the substance which reacts positively to the PAS treatment in oocytes or early embryonal cells was glycogen, because the reaction was negative when the oocytes were pretreated with diastase. The same finding was made in the present studies apply-

ing amylo-1, 4-α-1, 6-glucosidase. Thomson and Brinster (1966), Stern and Biggers (1969) and Ozias and Stern (1973) examined the decrease of glycogen content during the cleavage of mouse embryos and stated that the glycogen would be utilized as an energy source by the embryonal cells.

Recently, Tsafriri *et al.* (1972), and Marsh *et al.* (1972) assumed that the LH injected to the follicle stimulated cAMP production in the follicle cells and finally initiated the oocyte maturation even in the follicular environment. On the other hand, Cho *et al.* (1974) reported that the meiotic resumption of the oocytes *in vitro* was inhibited by the direct exposure to dbcAMP or theophylline and presumed that the increase of cAMP level in the oocytes might suppress the germinal vesicle breakdown which finally leads to the maturation division, because the agents are closely related to the elevation of the cAMP level. That is, dbcAMP penetated into the egg would have a connection to cAMP or mimic cAMP action and theophylline inhibits phosphodiesterase activity by which cAMP is converted to 5'-AMP, consequently these agents result in the increase of the cAMP level. Thus, both agents seem to be closely related to the concentration of cAMP in the cell.

Sutherland and Robison (1969) showed the initiation of glycogenolysis in the liver cells when cAMP concentration in the cell was increased. Thus, if the dbcAMP or theophylline is responsible for the increase in the intracellular level of cAMP, the glycogenolysis might take place in the oocytes.

Actually, in the present experiments, the breakdown of glycogen has been demonstrated in the oocytes treated with dbcAMP or theophylline. Based upon the above results, we presume that cAMP increased in the cell activates phosphorylase, and at the same time inactivates the glycogen synthetase, and consequently the amount of glycogen in the oocytes decreases. It is an interesting finding that the oocytes remained at the dictyate through the cultivation in the untreated medium still showed the initial amount of glycogen. This implies that the decrease of glycogen in the treated oocytes is only caused by the function of cAMP derived from dbcAMP or theophylline.

We assume that there are two factors to induce oocyte maturation, and these two must be occurred simultaneously. One is that the oocytes should keep enough amount of glycogen right before the meiotic resumption, and the other there must be an initiator for breakdown of glycogen at the earlier stage of meiosis. These assumptions are based upon the fact that the oocytes undergoing their maturation showed gradual decrease of glycogen as the division was progressed, the fact that the oocytes which have consumed glycogen during the culture in the presence of the agents restored glycogen just before starting meiotic division after transferred into the plain medium, and the fact that the oocytes arrested

spontaneously still kept glycogen unchanged in the untreated medium. However, still one question is remained; why are those oocytes consumed glycogen as seen in those proceeding meiotic division even though they are arrested in the presence of the agents? We assume that glycogenolysis in the untreated oocytes would occur by different way from those treated with dbcAMP or theophylline.

Cho and Yoo (1975) stated that the failure of oocyte maturation in the presence of dbcAMP or theophylline is solely due to the fact that the agent added to the medium suppresses the synthesis of RNA which is used to occur in the early stage of the meiosis, and induce specific proteins which are responsible for the breakdown of germinal vesicle, condensation of chromatin and/or formation of spindles.

If the excess amount of glucose was given to the medium containing inhibitory agent, the glycogenolysis in the oocytes was somewhat prevented, and the reason is uncertain. Furthermore the exogenous glucose given to the plain medium did not contribute for regaining of the glycogen by the oocyte once consumed during the cultivation. However, the amount of the glycogen restored in the oocytes reached to the level as high as those in the fresh one, and the arrested meiotic divison immediately enhanced. As the division was progressed, the level of glycogen gradually decreased as seen in the untreated oocytes. We can assume that the glycogen synthetase which has been inactivated by dbcAMP or theophylline becomes active upon the removal of the agents, and the glycogen would be synthesized. Until this time, the questions about the origin of the restored glycogen have not been solved. In other words, which is the basic substrate for the glycogen restoration; endogenous glucose most of which is arisen from the glycogen breakdown, or other carbohydrates such as pyruvate or lactate if the exogenous glucose is not utilized? Brinster (1969) and Ozias and Stern (1973) found that pyruvate was utilized for the glycogenesis in the embryonal cells *in vitro* instead of glucose. A group of the investigators stated that the nucleosides such as thymidine also became the precursor of polysaccharide (Piko, 1970) and amino acids could be used as the substitute for the energy materials for oocytes maturaion (Bae and Foote, 1975). Thus, if the exogenous glucose is not a source of the glycogen restoration, endogenous one, pyruvate or even bovine serum albumin supplemented to the medium would be utilized for its purpose. More detailed investigations for the answer are remained.

CONCLUSION

In the present experiments, it has been cleared that the glycogen is necessary for the resumption of oocyte maturation. However, the oocytes exposed to dbcAMP or theophylline failed to mature though the consumption of glycogen was made. The oocytes once showed the glycogenolysis by the agents could resume the

meiosis in the plain medium after the restoration of the glycogen at the same level as the fresh oocytes. The real mechanism and the source of the glycogen restoration are to be proved by further investigation.

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EXPLANATION OF FIGURE 3

A. The oocytes showing glycogenolysis during cultivation in the plain medium. Oocyte at dictyate stage (DIC) showed strong reaction to PAS-treatment, while as the oocytes at metaphase I-II showed weaker response (120X).

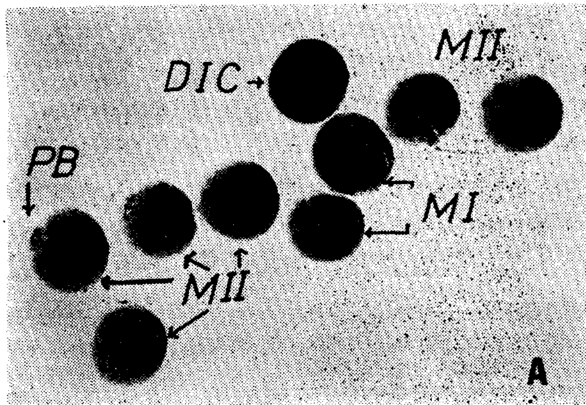
B. The oocytes, suppressed their meiotic resumption for 20 hours in the medium containing theophylline (100 $\mu g/ml$) showed lighter reaction to the PAS treatment. They kept germinal vesicles (GV) (120 X).

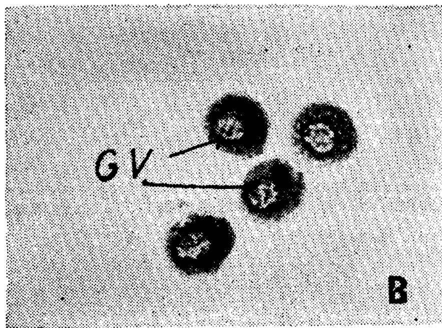
C. The oocytes with germinal vesicle(GV) but showing glycogenolysis. They were cultured in the medium containing dbcAMP (100 $\mu g/ml$) for 20 hours (120 X).

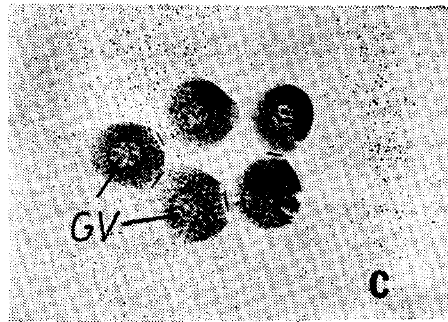
D. The ooytes showing strong (DIC) or weak (GVBD) reaction to PAS. They had been cultured in the medium of theophylline for 20 hours, and then transferred to the plain medium to show the restoration of glycogen which was consumed by the oocytes during the previous cultivation. Most of the oocytes started the meiotic resumption in 2 hours in the plain medium after restoration of the glycogen.

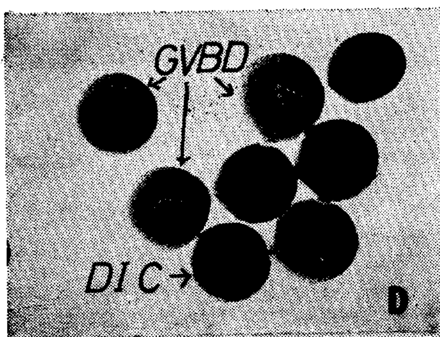
E. Some of the oocytes show a strong reaction to PAS, and the other a weak reaction. All of those, consumed glycogen in a medium with dbcAMP for 20 hours, were tranferred into the plain medium and cultured for another 2 hours. A part of the oocytes are keeping strong reaction to PAS (DIC) and the other on the meiotic division show a weak reaction (GVBD).

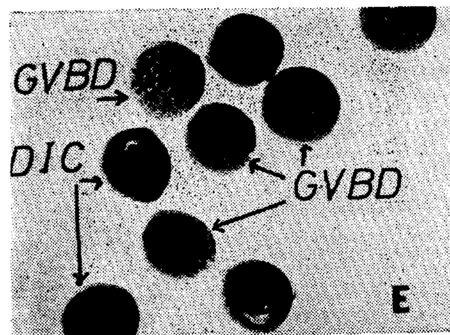
DIC : Dictyate oocyte
GV : Germinal vesicle
GVBD : Oocytes with brokendown germinal vesicle
MI : Oocytes at metaphase I
MII : Oocytes at metaphase II
PB : Polar body


DIC
MII
PB
MI
MII
A


GV
B


GV
C


GVBD
DIC
D


GVBD
DIC
GVBD
E