KOREAN JOURNAL OF ZOOLOGY
Vol. 27, No. 1, 1-12 (1984)

A Study of Alkaline Phosphatase Activity on the Preimplantation Mouse Embryos

Wan Kyoo Cho, Chung Choo Lee and Hae Kwon Kim
(Department of Zoology, College of Natural Sciences, Seoul National University)

초기 흰쥐 배아의 발생단계에 있어서의 Alkaline Phosphatase의 활성에 관한 연구

조 완 규·이 정 주·김 해 권
(서울대학교 자연과학대학 동물학과)

(Received September 15, 1983)

요 약

생쥐 난자 및 초기 배아의 alkaline phosphatase의 기능을 알아보기 위하여 생화학적인 방법으로 배아의 발생 단계에 따른 효소의 활성도를 측정하였으며, 동 효소의 저해제로 알려진 levamisole이 난자의 성숙분열 및 초기 배아의 발생에 미치는 영향을 관찰하였다.

1. 동 효소의 활성도는 4세포기에서 뚜렷이 나타나며, 각 발생단계에 따른 현저한 변화는 없는 것으로 나타났다.

2. Blastocyst의 alkaline phosphatase의 활성도는 1 mM 및 10 mM의 levamisole에 의해서 각각 40%와 70% 이상 억제되었다.

3. Levamisole은 0.5 mM 이상의 농도에서 난자의 극체 형성을 완전히 억제하였으며, 동일한 농도에서 2세포기 배아 및 morula의 퇴화현상을 일으켰다.

INTRODUCTION

Most mammalian eggs including human's reach the diplotene stage around the time of birth, thereafter they remain static until puberty. At puberty, the eggs that have progressed to mature within the follicle under precisely controlled hormonal conditions can be ovulated. However, the eggs can resume meiosis and form first polar body *in vitro* if they are liberated from the ovarian follicles into the culture medium (Baker, 1972).

In the maturation process are included dissolution of the nuclear membrane and nucleoli, chromosomal condensation, spindle fiber formation, and formation of the first polar body.

본 논문은 1982년도 문교부 학술연구조성비에 의하여 연구되었음.

Thus, numerous investigators have studied to clarify the mechanism of such meiotic maturation, investigating the effects of chemicals which induce or inhibit the meiotic division. Among these chemicals, for instance, dbcAMP inhibits germinal vesicle breakdown at diplotene stage reversibly (Cho *et al.*, 1974) and cytochalasin B inhibits meiotic maturation at metaphase I (Wassarman *et al.*, 1979). However, in spite of the many of the efforts, the mechanism of meiotic maturation is still unsolved.

During 24hr and 48hr after fertilization, mammalian eggs begin to cleave and become morulae, and then 24hr after morulae they grow to blastocysts (McLaren, 1972). Some workers have tried to explain the events taking place during the embryogenesis by untilizing the chemicals which affect the formation of microtubules or microfilaments (Siracusa *et al.*, 1980), and by comparing the changing patterns of various enzyme activities which are found in the early development (Schiffner *et al.*, 1976; Schultz *et al.*, 1977). Alkaline phosphatase (E.C.3.1.3.1.) is one of these enzymes. Since Izquierdo (1975) demonstrated that the enzyme activity first appears at the 4-cell mouse embryo, several studies have dealt with this enzyme in the preimplantation embryos. While Johnson *et al.*, (1977) suggested the first appearance of alkaline phosphatase at 8-cell embryos after the histochemical studies, Vorbrodt *et al.*, (1977) reported that the enzyme could be detected in the fertilized eggs and Mulnard and Huygens (1978) argued its first appearance at 2-cell stage of the embryo. In addition, the role of the alkaline phosphatase in the cell is not understood although its localization on the cell membrane is recognized. At present, the hydrolysis of phosphate esters (Rothstein and Meier, 1949) and transport of phosphate groups (Danielli, 1951) are generally accepted as its function, and other functions such as calcification, and transport of sodium, potassium, calcium and fat in the cells are now under investigation.

Here, the purpose of the present investigation was to study the alkaline phosphatase activities in the mouse oocyte and the preimplantation embryos by the biochemical method and to assess the effects of levamisole (L(−) 2, 3, 5, 6-tetrahydro-phenylimidazo (2, 1, -b) thiazole I) (Brunette *et al.*, 1981), which is known as an inhibitor to the alkaline phosphatase activity.

MATERIALS AND METHODS

1. Samples

Recovery of oocytes

Ovaries of ICR strain mice (3-4 weeks old) were dissected out and placed in the modified Krebs-Ringer bicarbonate solution (Biggers *et al.*, 1971) containing 100 μg/ml of dbcAMP (Sigma). Oocytes were liberated from the ovary by puncturing follicles with a fine needle under a dissecting microscope and those without cumulus cells but with visible germinal vesicles were selected and cultured or stored at −20°C.

Recovery of embryos

Embryos were obtained from ICR females which had been induced to superovulate with 0.5 I.U. PMSG (Sigma) and caged overnight with males. The presence of a vaginal plug in the next morning was taken as an indication of mating. Embryos were collected by flushing oviducts or uteri from mated females at the times appropriate and pooled for the culture or storage after washing.

2. Methods

Enzyme Assay

Assay procedures of alkaline phosphatase activity was performed with the biochemical method described by Bowers and McComb (1979) using p-nitrophenylphosphate as substrate. The oocytes were rinsed and disrupted by freezing and thawing for three times in 0.5 ml of 2-AMP buffer at pH 10.22 (2-Amino-2-methyl-1-propanol (0.89 M), HCl (0.2 M), Then 4.1 ml of the buffer, 0.2 ml of $MgCl_2$ (1.5 mM) and 0.2 ml of P-nitrophenylphosphate (225 mM) were added. The reaction mixture was incubated at 37°C for 2 hr in a shaker water bath. Levamisole, the enzyme inhibitor, was added to the culture medium with an appropriate concentration. After incubation, the samples were immediately assayed for determining the absorbance of liberated P-nitrophenol by the spectrophotometer (Bausch & Lomb) at 402.5 nm. The standard curve was performed with standards of P-nitrophenol, 0.625 to 10 μM.

Culture system

The culture work in the present studies was performed by microtube culture method (Cho, 1974). Oocytes or embryos were introduced into 10 μl medium previously set in a microtube (50 mm length, i.d. 1 mm), and incubated with 5% CO_2 in fully moistened air at 37°C. The details of the preparation for the culture tube were previously described (Cho, 1974).

Preparation of levamisole

Levamisole was dissolved in the medium at serial concentrations and the levamisole containing media was sterilized by millipore filter (0.45 μm pore size) before use.

At the end of culture periods, the samples were fixed with acetic alcohol and stained with 0.5% lacmoid for further observation of nuclear changes under a phase contrast microscope.

RESULTS

1. Activity of alkaline Phosphatase.

The size of samples for assessment of the enzyme activity in the oocytes and in the embryos was as follows; 200 oocytes, 200 of 2-cell embryos, 50 embryos each of 4-cell to 8-cell embryos, 10 of morulae and 10 of blastocysts. The enzyme activity of each sample expressed in P-nitrophenylphosphatase activity per egg or embryo per min. was shown in

Fig. 1. None or little enzyme activity was detected at the stage as earlier than 4-cell embryos, and from that stage onwards, the activity tended to keep a steady level. Activities of the enzymes in blastocysts were much higher than those of the enzymes in the earlier stages.

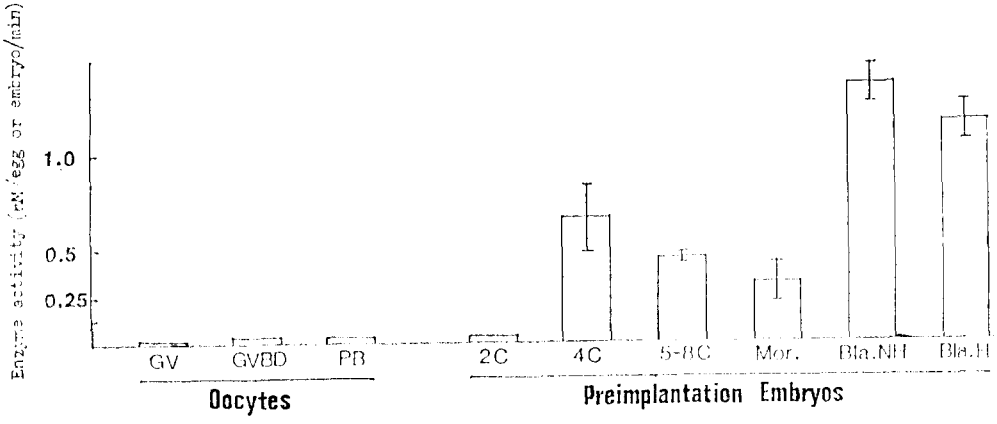

Fig. 1. Alkaline phosphatase activities in the oocytes and preimplantation embryos of mice. GV; Germinal vesicle, GVBD; Germinal vesicle breakdown, PB; Polar body, 2 C; 2-cell, 4 C; 4-cell, 5-8 C; 5-8 cell Mor.; Morula, Bla. NH; Not hatched blastocyst, Bla. H; Hatched bastocyst

2. Effect of levamisole on the activity of alkaline phosphatase.

Effect of levamisole on the enzyme activity by blastocysts recovered at 92~96 hr after HCG injection was measured. As shown in Fig. 2, levamisole inhibits the enzyme activity by 40% at 1 mM and 70% at 10 mM of the control.

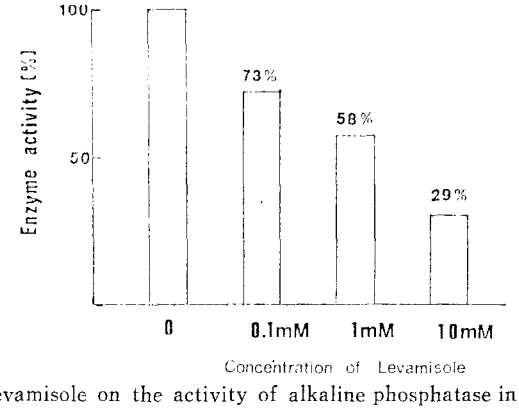

Fig. 2. The effect of levamisole on the activity of alkaline phosphatase in the mouse blastocysts.

3. Effect of levamisole on the oocyte maturation and early development.

Levamisole did not show any detectable effect on the germinal vesicle breakdown (GVBD) of the oocyte cultured *in vitro*, but it showed an inhibitory effect on the polar body formation of the oocyte (Table, 1), As seen in Table 1, it was found that the inhibitory

Table 1. The effect of levamisole on the maturation of mouse oocytes cultured *in vitro*.

Conc. of LM	4 hr culture			24 hr cultur				Total
mM	GV	GVBD	DEC	GV	GVBD	PB	DEG	
0	2	28		3	8	18	1	30
1.0	7	19	5	5	21		5	31
0.5	3	25	2	2	26		2	30
0.25	6	20	4	6	15	4	5	30
0.125	1	27	1	1	21	6	1	29

LM : Levamisole.
The dats shown here are sum of three repeated experiments.

Table 2. The effect of levamisole on the polar body formation by the oocytes with GVBD *in vitro*.

Conc. of LM	after 24 hr culture			Total
mM	GVBD	PB	DEG	
0	10	5		15
1.0	10		5	15
0.5	11	4		15
0.1	11	4		15
0.02	7	8		15
0.004	7	7	1	15

In this experiment, only oocytes with GVBD in culture for 4 hr were used.

effect increased with concentration of levamisole, and that the complete inhibition began at 0.5 mM of the chemical in the medium. In order to investigate the cause of this phenomena, eggs were fixed, stained and observed with the phase contrast microscope (Fig. 3, 4). It was found that, after 24 hr culture in medium containing levamisole, spindle fibers did not appeared. Also it was observed that the condensed chromosomes were scattered over the egg cytoplasm. And oocytes precultured in 1 mM levamisole medium for 24 hr also failed to complete metaphase II in plain medium for another 24 hr culture, producing reformed spindle fibers along the scattered chromosomes (Fig. 5). The same effect was examined in oocytes undergone already GVBD in 4 hr in plain medium. From Table 2, all the oocytes with GVBD failed to extrude polar bodies at a concentration of 1 mM levamisole.

The effect of levamisole on the *in vitro* development of 2-cell embryos recovered at 44-47 hr after HCG injection were examined by microtube culture. As shown in Table 3, the development of 2-cell embryos was delayed by increasing doses of the inhibitor in culture, and if the dose was above 0.5 mM, most of the 2-cell embryos degenerated in 72 hr culture. Moreover, when morulae were cultured for 24 hr in 1 mM levamisole medium, all the embryos degenerated (Table 4).

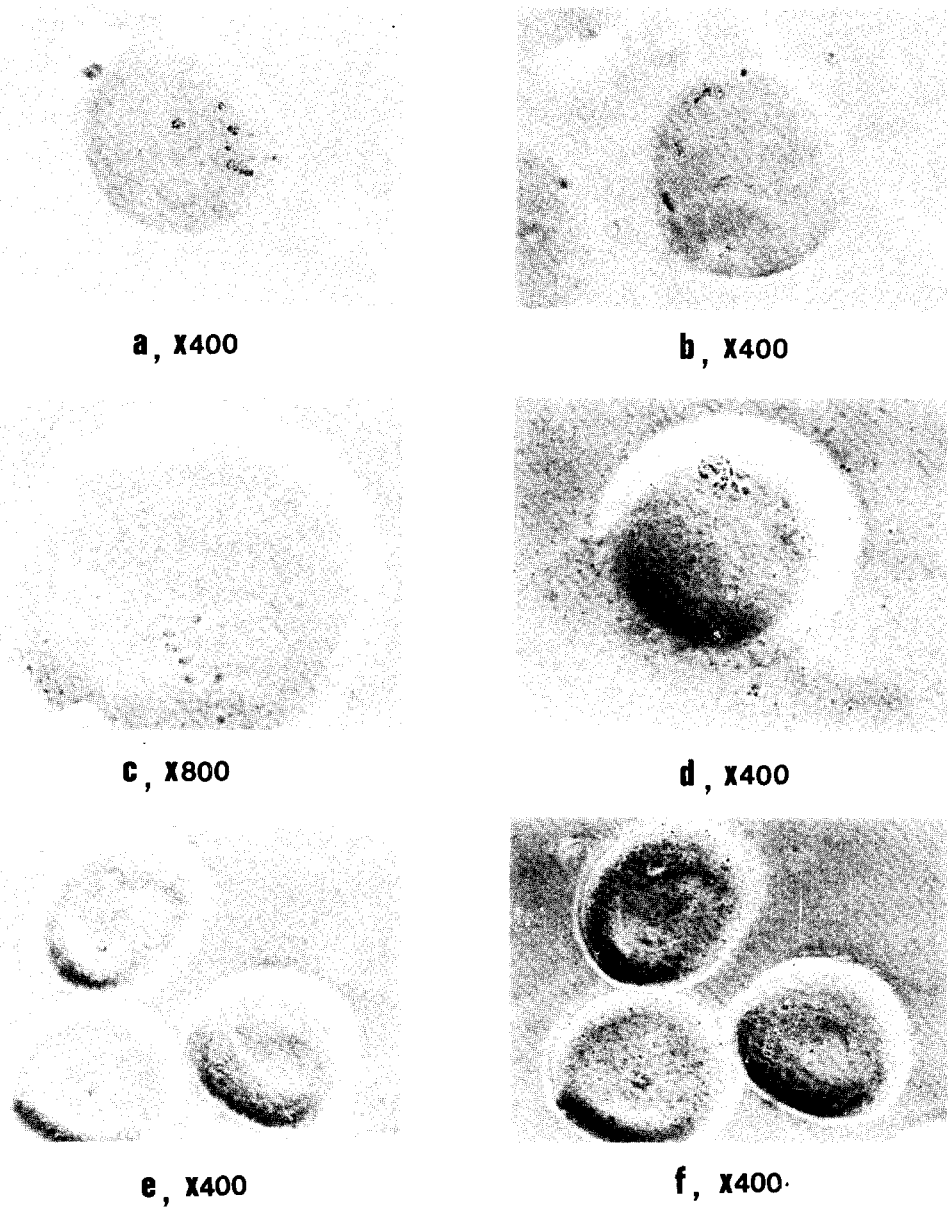

Fig. 3. Phase contrast micrographs showing oocytes cultured for 24 hr in medium containing 0.5 mM levamisole. Neither spindle nor polar body is shown in all the oocytes above.
a, b, c, f; oocyte with chromosomes scattered overall of the cytoplasm.
d; oocyte with gathered chromosomes at periphery of the egg.
e; oocytes with nuclear piknosis and condensed chromosomes.

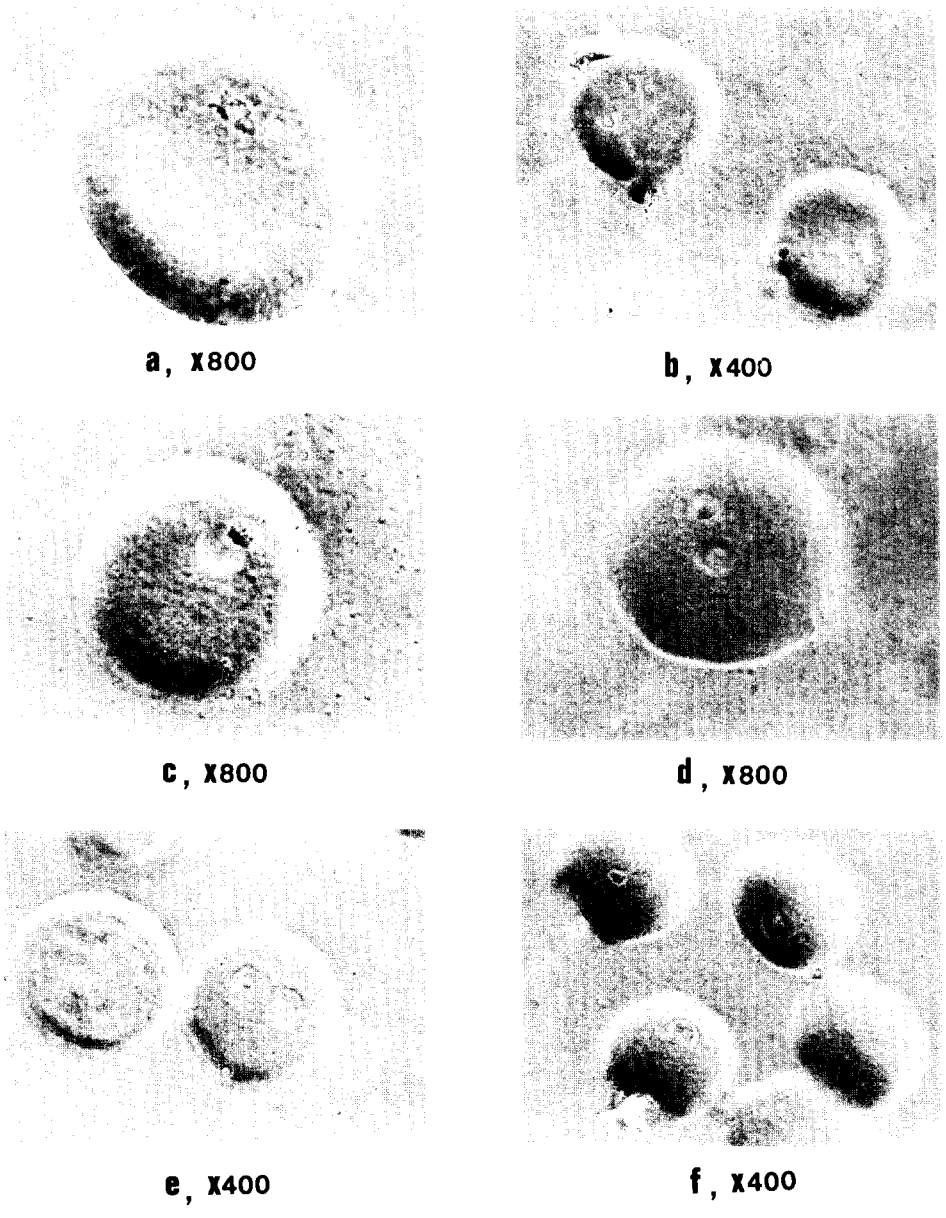

Fig. 4. Phase contrast micrographs showing oocytes cultured *in vitro* for 48 hr. Cocytes were cultured in medium containing 1 mM levamisole 24 hr and transferred to plain medium. All the oocytes are showing abnormal nuclear phases with absence of spindle fibers.
a, b: condensed chromosomes not showing meiotic apparatus.
c, d, e; oocytes showing abnormal nuclear phases. Condensed chromosomes are located in germinal vesicle-like structure.
f; oocytes showing abnormal nuclear phases.

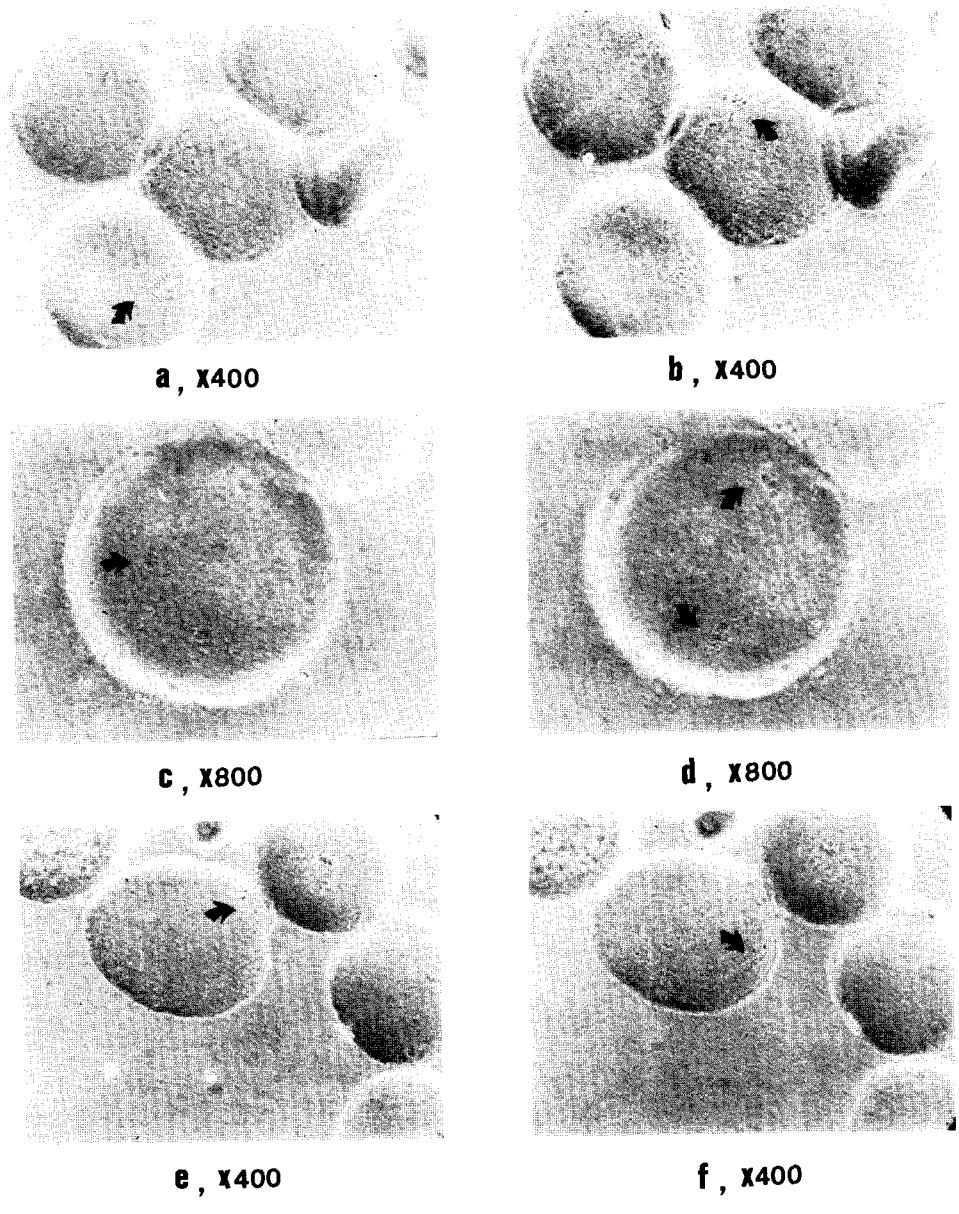

Fig. 5. Oocytes showing the reformed spindle fibers after 48 hr culture. Oocytes were cultured for 24 hr in medium containing 1 mM levamisole and then transferred to plain medium.
a, b; the same micrographs showing normal and abnormal rearrangements of spindle fibers respectively.
c, d; the same micrographs showing three different rearrangements of spindle fibers at the sites of scattered chromosomes.
e, f; the same micrographs showing two different rearrangements of spindle fibers at the sites of scattered chromosomes.

Table 3. The effect of levamisole on the clevage of 2-cell embryos cultured *in vitro*.

Conc. of LM	24 hr culture				72hr						Total
mM	2C	3-4C	5-8C	DEG	2C	3-4C	5-8C	Mor.	Hla.	DEG	
0	10	20	20		6	2	1		33	8	50
1.0	46	2		2	2					48	50
0.5	32	7	1		17	6				27	50
0.1	10	35	5		6	3		8	13	20	50
0.02	6	39	5		6	4	2		23	15	50

The data given are the sum of five repeated experiments.

Table 4. The effect of levamisole on the development of morulae cultured *in vitro*.

Conc. of LM	24hr culture			Total
mM	Mor.	Bla.	DEG.	
0		8		8
1.0			8	8
0.5		2	6	8
0.1		1	7	8
0.02		8		8
0.004		8		8

DISCUSSION

As previously described, it has been of the subjects of some controversy on the stages of the onset of alkaline phosphatase activity in the mouse embryos. Our results obtained by biochemical method are in accord with that of Ishiyama (1977) who found the first appearance of the enzyme activity at 4-cell embryos by the cytochemical method. It is uncertain, however, whether the appearance of enzyme activity means *de novo* synthesis from the activation of a pre-existent molecular species. Our results showed no significant difference in the level of enzyme activities among the stages of preimplantation mouse embryos. Hence, it seems that the alkaline phosphatase does not play any crucial role in the events like as compaction, cavitation and hatching in the embryos. However, the present experiments was designed to detect the effect of levamisole, which is one of the inhibitors of alkaline phosphatase activity, on the various stages of embryos *in vitro* as one of the indirect indicators for the assessment of alkaline phosphatase activity through embryogenesis.

Levamisole, a well known inhibitor of various mammalian alkaline phosphatases (McComb, 1979), inhibits canine bone, liver, kidney and placental alkaline phosphatases by 50% at 0.01 mM and 100% at 1 mM and canine intestinal alkaline phosphatase by 20% at

about 1 mM (Van Belle, 1972). Based on the results of our experiment that levamisole inhibited the enzyme activity of blastocyst by 50% at 1 mM, it is suggested that the role of alkaline phosphatase of early embryos would be different from those of differentiated tissues.

Levamisole did not exert any influence on GVBD of oocyte but inhibited the completion of metaphase II by leading the absence of spindle fibers and/or the abnormal localization of chromosomes. Moreover, the inhibitory effect of levamisole appeared in the oocytes already undergone GVBD in the previous culture in plain medium. Therefore, it is likely that levamisole inhibits rather the assembly of spindle fibers than GVBD or chromosome condensation. Thus, the abonrmal localization of chromosomes are likely due to the absence of spindle fibers. When only the oocytes undergone GVBD under the presence of levamisole were cultured extendedly in the plain medium for 20 hr or 36% of the oocytes in the former or 72% of those in the latter extruded polar body (unpublished). Thus, the oocytes once affected by levamisole have capability to reform the meiotic apparatus; spindle fibers. Some of oocytes with levamisole for longer hours, reformed spindle but they were located at the sites of chromosomes scattered over the egg cytoplasm. Hence, it seems that the reformed spindle fibers are primary spindles, i.e kinetochore fibers (Bajer and Mole-Bajer, 1970).

Based upon these various observations, we conclude that levamisole inhibits the assembly of spindle fibers of mouse oocytes reversibly. On the other hand, there are some reports on various drugs which block meiotic maturation at discrete stages reversibly prior to metaphase II *in vitro*. DbcAMP (Cho *et al.*, 1974) and chloroquine (Wassarman *et al.*, 1979) inhibit meiotic maturation at GV stage, whereas puromycin and colcemid (Wasserman *et al.*, 1976) circular bivalents (GVBD) stage and meiotic maturation can be blocked at metaphase I by cytochalasin B (Wasserman *et al.*, 1976), also it can be suppressed at anaphase I by prostaglandin (Cho, 1976). In contrast to the above reports and our results the effect of levamisole is similar to the effects of puromycin and colcemid except the different doses level of the agents to produce effectiveness, the former, 120 μg and the latter, 10 μg per ml.

In case of early development *in vitro*, levamisole induced degeneration or delayed development of 2-cell embryo and morula dependently to the dose given. According to the Siracusa (1980), colcemid and colchicine blocked development of 2-cell embryos, and the pattern of the action of these two chemicals and levamisole is similar to each other, as a spindle blocking agents, in spite of the extreme variation in effective doses between levamisole, 1 mM, and others, 0.1 mM.

ABSTRACT

In order to investigate the alkaline phosphtase activities in the mouse oocytes in matu-

ation and preimplantation embryos in developing in culture, the enzyme activities were measured by means of biochemical method. The *in vitro* effect of levamisole which is known as an inhibitor of the alkaline phosphatase was also observed on the oocyte in maturation and the embryos in early embryogenesis. The results obtained were as follows: The enzyme activity was not detected in the embryos unitl the stage of 4-cell, but it appeared first in the 4-cell embryos and the level of the activity was steady through up to the blastocyst. Levamisole inhibited the alkaline phosphatase activity in the blastocyst, and the activity decrased by almost 70% at 10 mM and 50% at 1 mM as compared with the control. In addition, levamisole inhibited completely the formation of polar body by the oocytes, and induced degeneration of the preimplantation embryos at the dose of 0.5 mM or higher.

REFERENCES

Bajer, A. and Mole-Bajer, J., 1970. Architecture and function of the mitotic spindle. *Adv. Cell Mol. Biol.* **1**; 213-267.

Baker, T.J., 1971. *In*; Reproduction in mammals, Vol. 1, p. 14, ed. Austin and R.V. Short, Cambridge Univ. Press.

Banks, E., S. Nakajima, L.E. Shapiro, O. Tilevitz, J.R. Alonzo and R.R Chianelli, 1977. Fibrous apatite grown on modified collagen. *Science* **198**:1164.

Biggers, J.D., Whitten and D.C. Whittingham, 1971. The culture of mouse oocytes *in vitro*. *In*: Methods in mammalian embryology, p. 86, ed. Daniel, J.C. Jr., W.M. Frenchman and Co.

Brinster, R.L., 1963. A method for *in vitro* cultivation of mouse ova from two cell embryo to blastocyst. *Exptl. Cell Res.* **32**:205-208.

Brunette, M.G., M. Chan and M. Lebrun, 1981. A microfluorometric method for alkaline phosphatase. *Anal. Biochem.* **115**:236-242.

Cho, W.K., 1974. A microtube culture method for mouse oocytes. *J. Reprod. Fertil.* **37**:437-440.

Cho, W.K. S. Stern and J.D. Biggers, 1974. Inhibitory effect of dbcAMP on mouse oocyte maturation *in vitro*. *J. Exptl. Zool.* **187**:383-386.

Danielli, J.F., 1952. Structural factors in cell permeability and secretion. *Symp. Soc. Exp. Biol.* **6**:1.

Ishiyama, V. and L. Izquierdo, 1977. The onset of phosphatase activity in early mammalian embryo. *J. Embryol. Exp. Morph.* **45**:305-308.

Izpuierdo, L. and P. Marticorena, 1975. Alkaline phosphatase in preimplantation mouuse embryos. *Exp. Cell. Res.* **92**:399-402.

Johnson, L.V., P.G. Calarco and M.L. Siebert, 1977. Alkaline phosphatase activity in the preimplantation mouse embryos. *J. Embryol. Exp. Morph.* **49**:83-89.

Kimber, S.J. and M.A.H. Surani, 1981. Morphogenetic analysis of changing cell associations following release of two cell and four cell mouse embryos from cleavage arrest. *J. Embryol. Exp. Morph.* **61**:331-331-345.

McComb, R.B., G.N. Bowers and S. Posen, 1979. *In*: Alkaline Phosphatase. p. 314. Plenum Press, New York.

McLaren, A., 1972. *In*: Reproduction in Mammals. Vol. 2, pp. 1-42 ed. C.T Austin and R.V. Short.

Cambridge Univ. Press.

Mulnard, J. and R. Huygens, 1978. Ultrastructural localization of nonspecific alkaline phophatase during cleavage and blastocyst formation in the mouse. *J. Embryol. Exp. Morph.* **44**:121-131.

Nagai, K., Izumi and H. Yoshida, 1966. Studies on potassium dependent phosphatase: Its distribution and properties. *J. Biochem.* **59**:295.

Rothstein, A. and R. Meier, 1949. The relationship of the cell surface to metabolism. IV. The role of cell surface phosphatase of yeast. *J. Cell Comp. Physiol.* **34**:97.

Schiffner, J. and H. Spiegelman, 1976. Fluorometric assay of the protein content of mouse and rat embryos during preimplantation development. *J. Reprod. Fert.* **47**:145-147.

Schultz, R.M. bnd P.M. Wassarman, 1977. Biochemical studies of mammalian oogenesis: Protein synthesis during oocyte growth and meiotic maturation in the mouse. *J. Cell. Sci.* **24**:167-195.

Siracusa, G., D.G. Whittingham and M. De Fellici, 1980. The effect of microtubule and microfilament disrupting drugs on preimplantation mouse embryos. *J. Embryol. Exp. Morph.* **60**:71-82.

Van Belle, H., 1972. Kinetics and inhibition of alkaline phosphatases from canine tissues. *Biochim. Biophys. Acta* **289**:158.

Vorbrodt, A., M. Konwinski, D. Solter and H. Koprowski, 1977. Ultrastructural cytochemistry of membrane-bound phosphatases in preimplantation mouse embryos. *Devel. Biol.* **55**:117-134.

Wassarman, P.M., W.J. Josefowicz and G.E. Letourneau, 1976. Meiotic maturation of mouse oocytes *in vitro*: Inhibition of maturation at specific stages of nuclear progression. *J. Cell. Sci.* **22**: 531-545.

Wassarman, P.M., R.M. Schultz, G.E. Letourneau, M.J. LaMarce, W.J. Josefowitz and J.D. Bleil, 1979. Meiotic maturation of mouse oocytes *in vitro*. *In*: Ovarian follicular and corpus luteum function, pp. 251-268, ed. A.R. Midgley and W.A. Sadler, Raven Press, New York.

Weisenberg, R.C., 1972. Microtubule formation *in vitro* in relation containing low calcium concentration, *Science* **177**:1104-1105.