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Effects of ionizing radiation on meiotic maturation of frog oocytes

II. *In vitro* studies

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SUMMARY

To investigate genomic activities in ovarian follicle cells and oocytes during meiotic maturation, ovarian pieces isolated from adult frogs (*Rana pipiens*) were irradiated with doses of X-ray ranging from 12000 to 96000 R and then treated with a pituitary extract and/or progesterone. When the ovarian tissue was treated with the pituitary extract alone or the pituitary extract plus a low dose of progesterone, irradiation with 12000 or 24000 R brought about an enhancement of ovulation, while irradiation with X-ray doses higher than 36000 R suppressed ovulation. On the contary, when the tissue was treated with high doses of progesterone, X-irradiation always suppressed ovulation. X-Irradiation at high dosages suppressed pituitary-induced maturation of oocytes with follicles, but not progesterone-induced maturation.

When oocytes irradiated with 48000 R or less were transferred into unirradiated female frogs, they proved to be fertilizable, but those irradiated with 72000 R or more were unfertilizable. Nuclear transplantation experiments revealed that X-irradiation did not deprive the oocytes of their capability to cleave, although it did retard the emergence of this capability in maturing oocytes. The embryos from oocytes irradiated with 48000 R or less developed into tadpoles, but no embryos developed beyond the gastrula stage if the oocytes were irradiated with 72000 R or more.

INTRODUCTION

Pioneering work of Heilbrunn and his colleagues (1939) revealed that excised ovarian pieces from a frog when immersed in a solution of pituitary extract underwent ovulation. Oocytes thus ovulated also proved to be fertilizable if they were passed through the oviduct of a gravid frog (Ryan & Grant, 1940). These techniques have been widely used for studying the mechanisms of ovulation of oocytes under the influence of pituitary gonadotropin (Wright, 1945, 1950, 1961). Mechanisms of governing oocyte maturation were also studied using these same techniques. It was found that an exposure of fully grown oocytes to the pituitary extract for a certain period of time induced germinal vesicle breakdown (GVBD) and that these oocytes later exhibited various characteristics of maturation, such as increased contractility of the surface, and capability of responding to activation stimuli. Furthermore, these oocytes successfully underwent cleavage when

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nuclei from blastulae were transplanted into them (Dettlaff, Nikitina & Stroeva, 1964).

On the other hand, it was found that progesterone not only enhances ovulation in the presence of pituitary extracts, but induces maturation even in the absence of pituitary action (Schuetz, 1967*a*). However, when oocytes were completely freed from their follicle cells, the pituitary extract did not induce maturation whereas progesterone was still capable of inducing maturation (Masui, 1967). Therefore, it is assumed that the pituitary gonadotropin stimulates the follicle cells to secrete a progesterone-like hormone which in turn induces maturation of the oocyte (Masui, 1967; Smith, Ecker & Subtelny, 1968).

Interestingly, the action of the pituitary gonadotropin was found to be sensitive to actinomycin D while that of progesterone was resistant (Dettlaff, 1966; Brachet, 1967; Schuetz, 1967*b*; Smith & Ecker, 1970). This may imply that genomic activities are involved in stimulation of the follicle cells by the pituitary gonadotropin but no genomic activities are obligatory for progesterone induced maturation of the oocyte. This viewpoint is supported by the result of the previous experiment (Masui, 1973), in which immature ovarian oocytes and mature uterine oocytes were irradiated with X-ray. It was found that destruction of the oocyte chromosomes by X-irradiation brought about no change in the morphology of androgenetic haploids developed from these X-irradiated oocytes whether X-irradiation took place before initiation of maturation or after completion of maturation.

Since X-irradiation of mother frogs at dosages higher than 36000R (1000 R = 0·258 C/1 kg air) caused a physiological disorder which prevents ovulation, the effect of higher doses of X-ray were studied *in vitro* using excised ovaries. If genomic activity of the follicle cells is prerequisite for pituitary-induced maturation of the oocyte, then X-irradiation of an isolated ovary should inhibit the maturation of these oocytes by suppressing the activity of the follicle cells. On the other hand, if genomic activity of the oocyte is not required for maturation, then X-irradiation of the ovary should have no effect on progesterone induced maturation. To test the validity of these predictions the excised pieces of ovary were irradiated with X-ray at dosages ranging from 12000 to 96000 R and then these irradiated tissues were treated with either the pituitary extract or progesterone to test their responsiveness.

MATERIALS AND METHODS

Mature male and female *Rana pipiens* obtained in early winter from Minnesota and Wisconsin, U.S.A., were used throughout these experiments. They were kept at 4–5 °C until use. Experiments were carried out during the period between February and June. X-Irradiation was carried out with a Maxtron 250-X-ray (General Electric). Doses delivered were varied ranging from 12000 to 96000 R at a dose rate of 2000 R per min (250 kV, 30 mA, HVL 2·25 mm Al, filter

0·25 mm Al). The conditions for irradiation in this experiment are the same as described in the previous experiment (Masui, 1973). During the period of X-irradiation excised pieces of ovarian tissue, each containing approximately 30 oocytes, were kept at approximately 4 °C in a Petri dish containing ice-chilled frog Ringer's solution.

Follicle-free oocytes were prepared by peeling off the follicular envelope manually with forceps after 30 min treatment with Ca–Mg-free EDTA-containing Ringer's solution (Masui, 1967). The X-irradiated oocytes either with or without the follicle were transferred into a Ringer's solution containing pituitary suspension or progesterone and kept at 18 °C for 48 h. The solution contains pituitary homogenate at a ratio of one pituitary per 20 ml. Progesterone was added at various concentrations ranging from 0·1 to 10 μg per ml.

The germinal vesicle breakdown (GVBD) was examined by dissecting oocytes after fixation in 5 % formalin. Maturity of these oocytes was tested by either inseminating them after passage through the oviduct of foster mothers or by transplantation of a blastula nucleus. For identification of transferred oocytes they were stained with Ringer's solution containing Nile blue sulfate (0·02 %) or neutral red (0·01 %) for 10 min. Transferring oocytes into the body cavity of a foster-mother frog, nuclear transplantation and chromosomal examination were all carried out according to the procedures described in the preceding paper (Masui, 1973). The embryos were reared in 10 % Ringer's solution at 18 °C.

RESULTS

Effects of X-ray on ovulation and GVBD

The results are represented in Figs. 1 and 2. The percentage of oocytes ovulated from unirradiated ovarian pieces was usually less than 10 %, giving an average of 7 %, if they were treated with a pituitary homogenate solution. However, addition of progesterone significantly increased the frequency of ovulation with increasing steroid concentration. Furthermore, a high concentration (10 μg/ml) of progesterone induced ovulation in the absence of pituitary homogenate in some experiments carried out after February. Dissection of oocytes after fixation with formalin revealed that many oocytes, either ovulated or unovulated, had undergone GVBD. The frequency with which the oocytes underwent GVBD was increased with increasing concentration of progesterone. When the follicle was removed GVBD took place in all the oocytes.

X-Irradiation of ovarian pieces before hormonal treatment resulted in a remarkable change in the frequencies of both ovulation and maturation. As seen in Fig. 1, the ovarian tissue irradiated with 12000 or 24000 R ovulated more frequently than those unirradiated if the tissue was treated with pituitary suspension alone or with pituitary suspension plus a low dose (0·1 μg/ml) of progesterone. However, as the dosage of X-ray was increased higher than 36000 R, the frequency of ovulation decreased. On the other hand, when ovarian tissues

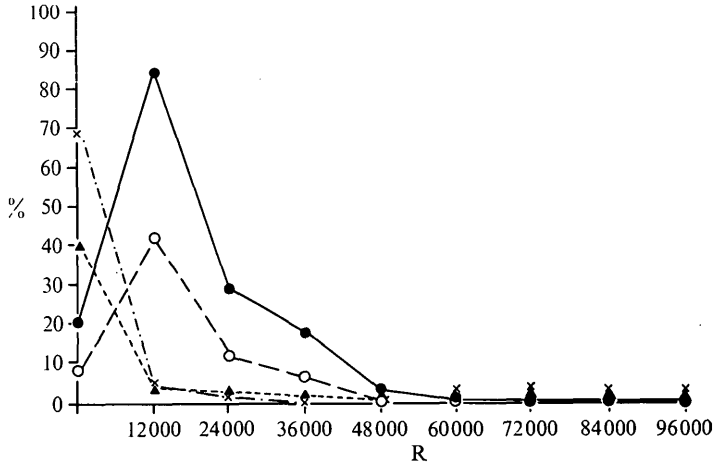

Fig. 1. Effect of X-ray on ovulation. Ordinate: percentage of ovulated oocytes calculated from four different experiments. In each experiment excised ovarian pieces which contain about 30–50 oocytes were irradiated before hormone administration. Abscissa: doses of X-ray. ○, pituitary suspension; ●, pituitary suspension + progesterone (0·1 μg/ml); ×, pituitary suspension + progesterone (1·0 μg/ml); ▲, progesterone alone (10 μg/ml).

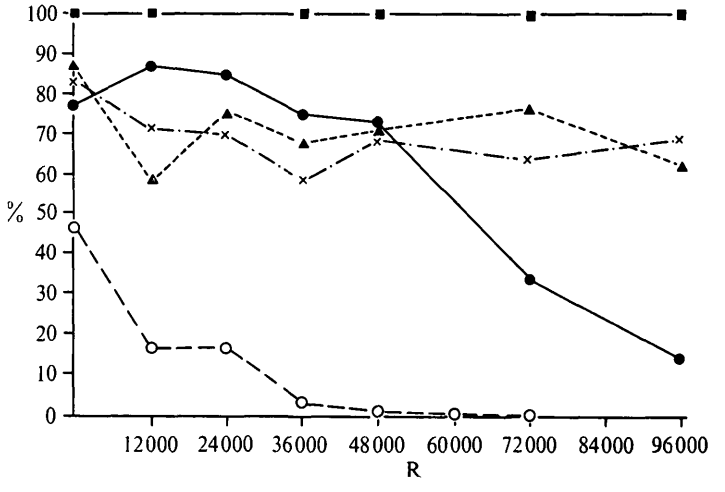

Fig. 2. Effect of X-ray on maturation. Ordinate: percentage of germinal vesicle breakdown calculated from four different experiments. In each experiment excised ovarian pieces which contain about 30–50 oocytes were irradiated before hormone administration. Abscissa: doses of X-ray. ○, pituitary suspension; ●, pituitary suspension + progesterone (0·1 μg/ml); ×, pituitary suspension + progesterone (1·0 μg/ml); ▲, progesterone alone (10 μg/ml); ■, follicle-free oocytes treated with progesterone (1·0 μg/ml).

were treated with higher doses of progesterone the percentage of ovulated oocytes was always found to decrease progressively with increasing dose of X-ray. On the other hand, as seen in Fig. 2, the percentage of oocytes which underwent GVBD was progressively decreased with increasing dose of X-ray

Table 1. *Effects of X-ray on maturation of oocytes* in vitro *tested by insemination*

(Oocytes having been maturing *in vitro* after progesterone treatment were transferred into foster-mother frogs to invest the oocyte with the jelly for insemination. Percentages of the eggs fertilized were calculated against the number of the oocytes recovered without damages.)

Host frog	Time of oocyte transfer (h)*	Time of insemination (h)*	Dose of X-ray (R)	Staining of oocytes	Oocytes transferred	Oocytes recovered without damage	Activation (%)	Cleavage (%)	Blastula (%)
D_1	18	48	0	Blue	60	56	52	39	37
			0	Red	60	32	63	31	31
D_2	24	48	0	Blue	110	91	73	56	54
			0	Red	75	57	68	68	68
D_3	30	72	0	Red	130	97	94	70	63
			0	Blue	130	71	86	82	76
Total					564	404	61	49	46
E_1	32	48	12000	Blue	100	53	53	42	42
			0	Red	100	69	62	43	43
E_2	30	54	12000	Blue	100	73	92	78	74
			0	Red	100	79	95	73	71
F_1	30	54	24000	Red	100	51	80	53	53
			48000	Blue	100	30	50	20	20
G_1	32	56	72000	Blue	150	80	5	1	0
			0	—	—	—	—	—	—
H_1	32	56	96000	Blue	100	22	100	0	0
			0	Red	100	60	82	57	53

* Hours after hormone administration.

when the ovarian tissues were treated with pituitary suspension alone or pituitary suspension plus a low dose of progesterone (0·1 μg/ml). However, no decrease in the percentage of GVBD was brought about by X-irradiation if high doses of progesterone were administered.

Summarizing the results described above it becomes clear that ovulation induced under the influence of either pituitary gonadotropin or progesterone is generally suppressed by X-irradiation, although its enhancement was evident when the ovarian tissues having been irradiated with low doses of X-ray (12000–24000 R) were treated with pituitary suspension lacking progesterone or containing a small dose of it. Induction of GVBD with pituitary gonadotropin in the presence of follicle cells is also suppressed by X-irradiation, while progesterone-induced GVBD is not.

Test of maturity of X-irradiated oocytes by means of fertilization

Follicle-free oocytes treated with progesterone after X-irradiation were stained with the vital dye Nile blue sulfate. Unirradiated oocytes, taken from the same donor frog and treated with progesterone, were stained with neutral red and both X-irradiated and unirradiated oocytes were simultaneously transferred into a female injected with pituitary suspension and progesterone 24 h in advance. For treatment of the oocytes with progesterone they were exposed to a progesterone solution of 10 μg/ml for 20 min. Transfer of the oocytes was carried out 18, 24 or 30 h after progesterone treatment.

The results of a control experiment in which unirradiated oocytes having been stained with either Nile blue sulfate or neutral red and simultaneously transferred into a primed female showed that both classes of oocytes underwent cleavage with almost the same frequency and developed similarly, irrespective of the dye used to stain them (Table 1, D_{1-3}). It can be safely assumed that staining with different dyes does not significantly influence the fertilizability of these oocytes. In addition, there was no sign of mixing of the two colors in any oocytes when they were squeezed out of the uterus of the host, therefore, it is also safe to rely upon this technique of staining with the two dyes to differentiate the X-irradiated oocytes from those unirradiated.

The fertilizability expressed as the percentage of oocytes undergoing cleavage after insemination was variable among different recipient frogs, ranging from 30 to 80 % in the cases in which unirradiated oocytes of two different colors were transferred. Unirradiated oocytes transferred together with those X-irradiated also showed similar variation in fertilizability, ranging from 40 to 70 %. Oocytes X-irradiated with lower doses such as 12000 or 24000 R showed a fertilizability which fell in this range of variability. Moreover, the fertilizability of such X-irradiated oocytes was found to be almost the same as that of the unirradiated oocytes which were transferred together with the X-irradiated oocytes into the same recipient (Table 1, E_1 and E_2).

However, when oocytes were irradiated with higher dosages of X-ray such as 48000 R or more, the fertilizability was significantly decreased. For example, in an experiment where oocytes irradiated with 24000 R and those with 48000 R, both of which had been isolated from the same piece of ovary, but stained in different colors, were transferred into the same recipient after progesterone treatment, it was found that the fertilizability of those irradiated with 48000 R was significantly lower than those irradiated with 24000 R (F_1 in Table 1). None of the oocytes irradiated with 72000 or 96000 R were fertilizable except for one which underwent abnormal cleavage, while unirradiated oocytes taken from the same donor and transferred into the same recipient were fertilized with a considerably high frequency.

From the above experiments it is concluded that X-irradiation of the oocytes with 12000 or 24000 R does not interfere with the maturation process of the

Table 2. *Effect of X-ray on maturation* in vitro *tested by nuclear transplantation*

Dose of X-ray (R)	Time of nuclear transplantation (hours after hormone administration)							
	48 h				68 h			
	Oocytes examined	Activation (%)	Cleavage (%)	Blastula (%)	Oocytes examined	Activation (%)	Cleavage (%)	Blastula (%)
0	70	77	46	27	85	97	48	35
12000	72	78	46	31	63	97	60	33
24000	88	76	38	20	75	92	65	47
48000	64	67	33	19	63	81	51	44
72000	62	40	10	3	55	71	60	46
96000	79	42	5	3	73	40	19	14

oocytes induced *in vitro* when the maturity of these oocytes was tested by fertilization, whereas X-irradiation with 48000 R or more decreases fertilizability of these oocytes.

Test of maturity of the X-irradiated oocytes by nuclear transplantation

Follicle-free oocytes dissected from ovarian pieces which had been irradiated with different dosages of X-ray were treated with a progesterone solution in the same way as in the preceding experiment. A nucleus from blastulae or early gastrulae was transplanted into the oocyte 48 or 68 h after progesterone treatment. Enucleation of the recipient was avoided. The experiment was carried out with oocytes from the same frog from which 20 or 25 oocytes were subjected to the same treatment at one time and the experiment was repeated using four different females. A summary of these results is given in Table 2.

A majority of X-irradiated and progesterone-treated oocytes after transplantation of a nucleus showed the characteristic signs of activation such as loss of surface gloss, which took place immediately after penetration of the needle, and later puckering of the surface or the formation of a cleavage furrow. Many of these oocytes underwent cleavage to form a blastula as shown in Fig. 3. When nuclear transplantation was carried out 48 h after progesterone treatment activation took place with similar frequency in both X-irradiated and unirradiated oocytes until the dosage of X-ray reached 48000 R. However, when oocytes were irradiated with 72000 or 96000 R a significant decrease in the percentage of activated oocytes was observed. When oocytes were irradiated with 48000 R or less, half of the activated oocytes were found to undergo cleavage, but a gradual decrease in the percentage of cleaving oocytes was brought about with increasing dose of X-ray. Oocytes irradiated with higher doses failed much more frequently to cleave even if they were activated.

On the other hand, if oocytes received a transplanted nucleus 68 h after

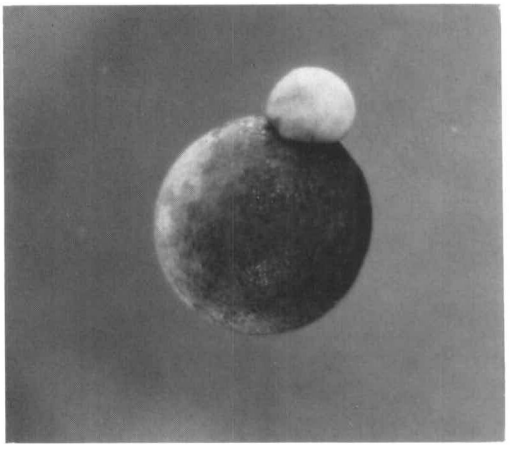

Fig. 3. An X-irradiated oocyte undergoing cleavage after receiving nuclear transplantation from a blastula. The oocyte was irradiated with 72000 R and treated with progesterone (10 μg/ml for 10 min). × 15.

progesterone treatment, it was found that there was an increase in the frequency with which oocytes underwent cleavage as well as activation irrespective of the dosage of X-ray with which the oocytes were irradiated. It was noted that no decrease in the frequency of cleavage was observed in the oocytes irradiated with X-ray as compared with those unirradiated, but rather there appears to be a significant increase of successful cleavage in X-irradiated oocytes except for those irradiated with 96000 R. In this latter case, a significant decrease in the frequency of cleavage was found even though an apparent improvement took place in the cleavage capability of the oocytes when compared with those having received a nuclear transplantation 48 h after progesterone treatment.

From the above results it seems likely that the capability of X-irradiated oocytes to undergo cleavage is increased during the process of incubation from 48 to 68 h after progesterone treatment and reaches that of unirradiated oocytes, if X-ray delivered was 72000 R or less, as best exemplified in the oocytes irradiated with 72000 R. Only the oocytes irradiated with 96000 R still showed a significantly lower level of cleavage capability as well as activatability. Whether these low capabilities of the oocyte irradiated with the heaviest dose of X-ray can be recovered with further incubation remains a matter of question at the present.

Development of the X-irradiated oocytes

In the insemination of X-irradiated oocytes after the passage through the oviducts of a foster-mother frog, only the oocytes irradiated with 48000 R or less were found to be fertilizable. The oocytes which had started cleaving usually reached the blastula stage, exhibiting no cleavage abnormalities such as formation of blastomeres of extremely unequal size or partial cleavage (Table 1). All blastulae also started gastrulation. However, as shown in Table 3, some of them

Table 3. *Effect of X-ray on the development of the oocytes matured* in vitro

(Oocytes having been maturing *in vitro* after progesterone treatment were transferred into foster-mother frogs to invest the oocyte with the jelly and inseminated. Percentages of the embryos developed were calculated against the total number of blastulae obtained.)

			Development of oocytes (%)					
Host frog	Dose of X-ray (R)	Total no. of blastulae	Failure in gastru-lation	Arrested at neurula stage	Arrested at tail-bud stage	Abnormal tadpoles	Haploid tadpoles	Diploid tadpoles
D_1, D_2, D_3	0	185	4	2	16	19	0	59
E_1, E_2, H_1	0	118	8	14	4	14	0	60
E_1, E_2	12000	77	14	27	0	29	39	0
F_1	24000	27	30	40	0	30	0	0
F_1	48000	6	0	0	0	100	0	0

failed to complete gastrulation so that further development was severely disturbed by extrusion of a large amount of yolk or by failure to form the neural plate. Of unirradiated oocytes less than 10 % suffered from failure in gastrulation. The percentage of oocytes failing gastrulation is increased with increasing dose of X-ray with which the oocytes were irradiated, but all the oocytes irradiated with 48000 R passed the gastrula stage. However, it is premature to state that the success in gastrulation of these heavily irradiated oocytes is a rule in view of the scarce number of specimens observed.

Some of the embryos having passed gastrula stage and completed formation of the neural tube were arrested before growth of the tail-bud or before hatching. These abnormal embryos occurred in both unirradiated and X-irradiated groups of oocytes with considerable frequency. Most of the embryos having passed gastrulation reached tadpole stages, although many of them showed abnormalities in their morphology such as a crooked body, edema and bifurcated tail which were described previously (Masui, 1973). All the tadpoles without such abnormalities exhibited diploid characteristics if they were not irradiated, whereas all the tadpoles which developed from the oocytes irradiated with 12000 R invariably showed the characteristic haploid syndrome as described in the previous paper (Masui, 1973). All the tadpoles developed from the oocytes irradiated with 24000 or 48000 R were abnormal with respect to their external morphology. Chromosome examination carried out with tail-tip squash specimens of the tadpoles taken randomly out of each group of X-irradiated and unirradiated oocytes confirmed the judgement of the ploidy that was reflected by their external characteristics. Thus, it can be stated that all the embryos which developed from the oocytes irradiated with 12000 R or more and reached the tadpole stage totally lacked the maternal chromosome complement.

Development of the oocytes which had a nucleus transplanted into them usually failed in gastrulation although more than half of the transplants started cleavage and reached the blastula stage (Fig. 3). Whether they were X-irradiated or not, and irrespective of doses of X-ray with which the oocytes were irradiated, failure in gastrulation took place with almost the same frequency. None of the embryos completed gastrulation. Some of them developed into abnormal tail-bud, but no tadpoles developed.

DISCUSSION

X-Irradiation of pieces of ovarian tissue with 12000 or 24000 R caused enhancement of ovulation when ovulation was induced under the predominant influence of pituitary gonadotropin. However, higher dosages of X-ray above 36000 R invariably suppressed ovulation. It is interesting to note that in mice enhancement of ovulation was also observed after whole-body irradiation with a fairly strong dose of X-ray such as 600 to 1500 R (Hahn & Ward, 1967). Although no definite interpretation has been given for this effect, it was speculated that enhancement of circulation by increasing intravascular space of the ovary is a probable cause for the enhanced ovulation (Hahn & Ward, 1971). Inasmuch as enhancement of ovulation by X-irradiation with isolated frog ovary *in vitro* was brought about by a far stronger dose of X-ray than that with mouse ovary *in vivo*, direct comparison of the effect of X-ray in these two animals appears to have no valid theoretical basis. Nevertheless, it is a temptation to assume that a common mechanism operates in the enhancement of the ovulatory function by X-irradiation in both animals.

It is well known that progesterone stimulates ovulation in amphibians (Zwarenstein, 1937; Burgers & Li, 1960; Wright, 1961). The present experiment confirms this. Moreover, a recent study with cyanoketone, a chemical analogue that inhibits 3β-hydroxysteroid dehydrogenase showed that inhibition of this enzyme leads to inhibition of ovulation (Wright, 1971). The author suggested that pituitary hormone induces production of progesterone in the follicle which in turn causes ovulation. If so, it is speculated that X-irradiation at relatively low doses might facilitate production of progesterone by follicle cells.

Induction of oocyte maturation by pituitary gonadotropin is a rather radio-resistant process in view of the fact that it was not completely prevented even after X-irradiation of the ovary with 48000 R. This observation confirmed the report by Dettlaff (1967), who irradiated ovarian oocytes with 50000 R. However, X-irradiation with higher doses completely suppressed maturation of oocytes induced by pituitary gonadotropin whereas no suppression of the maturation induced with progesterone was observed at any dose of X-ray examined. This indicates clearly that the modes of action of the two hormones in the induction of maturation are different. Since the pituitary gonadotropin does not induce maturation of oocytes if the follicle was removed, it can be assumed that the pituitary gonadotropin acts on the follicle which in turn exerts its influence

on the oocyte to induce maturation (Masui, 1967). From this assumption it seems likely that high dosages of X-ray acts on the follicle cells to prevent them from responding normally to the action of pituitary gonadotropin, but it does not inhibit the mechanism in the oocyte to respond to progesterone. That is, the process of maturation itself is entirely resistant to X-ray.

It was found that heavily irradiated oocytes, in which maturation was induced *in vitro* by progesterone treatment, developed after fertilization or nuclear transplantation. The oocytes irradiated with 48000 R or less were found to be fertilizable if they were coated with normal jelly following passage through foster-mother oviducts, and many of them developed beyond gastrulation to reach tadpole stages. However, no fertilization took place when oocytes were irradiated with 72000 R or more, although these oocytes were found to cleave after nuclear transplantation. It is conceivable that irradiation with such heavy doses of X-ray may damage mechanisms which are necessary for making the surface of these oocytes accessible to the sperm. On the other hand, nuclear transplantation experiments revealed that when transplantation was carried out 48 h after progesterone treatment, more X-irradiated oocytes failed to cleave than those unirradiated, and the percentage of oocytes which started cleavage was progressively decreased with increasing dose of X-ray. However, the percentage increased during incubation from 48 to 68 h after progesterone treatment and even surpassed the level of the unirradiated oocytes in most cases. Therefore, it seems very likely that X-irradiation causes a delay in the maturation process but not an irrevocable inactivation of the oocytes.

From this result it is concluded that maturation of the oocyte is a process highly resistant to ionizing radiation as far as the maturity of the oocyte is judged from its capability to cleave. This may imply that gene activity of the oocyte nucleus is neither obligatory for completion of maturation of oocytes nor for acquisition by the oocytes of the capability to cleave. This is also suggested by the fact that actinomycin D does not inhibit maturation induced by progesterone (Schuetz, 1967*b*; Smith & Ecker, 1970), while it inhibits pituitary-induced maturation (Dettlaff, 1966; Brachet, 1967). However, it should also be noted that oocytes frequently failed in development beyond gastrulation when they were irradiated with doses higher than 24000 R. Since elimination of chromosomes by irradiation with 24000 R or less gave rise to complete development of haploid tadpoles after fertilization, the failure in development beyond cleavage stage after irradiation with higher doses of X-ray appears to be caused by radiation damage to the cytoplasm of the oocyte.

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