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ANALYSIS OF X-RAY-INDUCED MITOTIC DELAY IN SEA URCHIN EGGS

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Bhashini Rao

(Ph. D. Thesis)

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ANALYSIS OF X-RAY-INDUCED MITOTIC DELAY IN SEA URCHIN EGGS

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ABSTRACT

x-Ray-induced reversible mitotic delay in sea urchin eggs was studied. Detailed cytological analysis showed that sublethal doses of x rays affected the process of chromosome condensation in these eggs. The delay in chromosome condensation corresponded directly to the delay in cell division.

Rate and time course of DNA synthesis in these cells were studied over two division cycles by following the incorporation of tritium-labeled thymidine. The study of radiosensitivity of DNA synthesis showed that x-ray doses that caused reversible mitotic delay had no effect on the time or rate of DNA synthesis in these cells. Furthermore, the experiments showed that delay of DNA synthesis in the second division was a result of interference with mitosis. An equation to determine the S period was derived and used to compute the S period in these cells.

Variation of mitotic delay with dose and time of irradiation was studied. There existed in prophase a critical point. If cells were irradiated before this point, there was a delay in the first cleavage. Cells irradiated after this point did not show any delay in the first cleavage, but the second cleavage was delayed. Shortly before the onset of the visible prophase, there was a stage in which the delay produced by a given dose was maximum.

Some experiments showing the effect of halogenated pyrimidines on cell division and the modification of this effect by radiation were described.

I. PURPOSE OF THE STUDY

Irradiation-induced mitotic inhibition has been studied in a variety of experimental materials, but the mechanisms underlying this inhibition are still very poorly understood.

One disadvantage in many of these previous studies has been the use of high doses of x rays, which has made it difficult to distinguish between reversible and irreversible damage.

The most direct approach to the problem of the effect of low doses of x rays on mitosis is to study either individual cells or synchronous populations of cells. This allows one not only to irradiate cells at a particular stage of division but also to study which cytological stages or biochemical functions may be affected. Apart from the studies on grasshopper neuroblasts by Carlson (Carlson, 1940, 1942, 1950, 1954), and by Henshaw (1940) and Yamashita et al. (1939) on Arbacia eggs, there are very few investigations on mitotic inhibition in relation to the various events at the cellular level.

There is little doubt that cytological effects of x-irradiation are the results of chemical and physical changes, but it is important first to determine what function, at the cytological level, is the target of antimitotic action of x rays.

As Mazia (1961) points out: "There are numerous preparations for a given division, all of which must be completed before the mitotic apparatus goes into action. The preparations may take place in parallel as well as sequentially." Some of these functions can be recognized at the cytological level.

The purpose of the study presented here was (a) to investigate in detail the effect of sublethal doses of x rays at the cytological level, and (b) to study the possible biochemical mechanisms underlying the division delay.

II. REVERSIBLE MITOTIC DELAY AS RELATED TO CHROMOSOME CONDENSATION

A. Cytological Study of Division Delay

In order to gain some understanding of radiation-induced division relay, it is essential to focus our attention on the known major events of cell division. Various biochemical, physical, and structural changes are taking place throughout the division cycle of the cell (Mazia, 1961; Celfant, 1963). Some of these changes are taking place simultaneously whereas others occur in sequence and are interdependent. It is also possible, by using specific inhibitors, to dissociate many of these events. For example, reproduction of the mitotic centers in sea urchin eggs can be blocked without any interference with DNA synthesis (Bucher and Mazia, 1960). Kanzir and Errera (1954) show that in E. coli, DNA synthesis precursors accumulate even though DNA synthesis is inhibited. Celfant and Clemmons (1955), working with epithelial cells of rat uterus, report the synthesis of nonhistone chromosomal protein in the absence of any DNA synthesis. Many more examples of this type can be found in the literature.

Blockage of cell division can be accomplished by interfering with any one of these changes.

At the cytological level a number of events that occur during the process of cell division can be recognized —formation and separation of the asters, disappearance of the nucleolus, breaking of the nuclear membrane, condensation and movement of the chromosomes, formation of the cleavage furrow, and finally uncoiling of the chromosomes and reformation of the nucleolus and the nuclear membrane.

The investigation reported in this chapter was undertaken in an effort to study the effect of sublethal doses of x rays on some of these processes, and to relate this to the process of reversible mitotic delay.

For the purpose of this work an operational definition has been given to the term chromosome condensation, without going into the actual mechanism of chromosome condensation. The beginning of the prophase is often recognized by the fact that the chromosomes can be resolved as microscopic threads. The same criterion is used for the process of chromosome condensation. It is well established that condensation of the chromosomes continues through metaphase (Bajer, 1959), but here we are more interested in the visible onset of the condensation process, i.e., the time at which the chromosomes are first visible under the phase microscope.

1. Materials and Methods

The common Pacific Coast purple sea urchin, *Strongylocentrotus purpuratus*, was used as the source of eggs and sperm. This species is usually fertile from October to April and capable of giving off large numbers of eggs or sperm. Spawning was induced by injecting about 2 ml of 0.5 M KCl solution into the body cavity of the urchin. The eggs were collected in sea water at 5°C and the sperm was collected in dry syracuse dishes. The eggs were washed with filtered sea water by means of settling, decanting, and passing the eggs once through nylon mesh.

The irradiation was carried out with 50-kV x rays at a dose rate of 52 r/sec. The source of x rays was a Machlett OEC-60 beryllium-window x-ray tube. This tube is specially suitable because of the radial symmetry of its x-ray beam and the extremely high dose rates obtainable. The largest dose employed, 2000 r, required only about 40 sec to administer. This time interval is very small compared with the division time of these cells.

The irradiation of the eggs, sperm, or zygotes, as the case might be, was done after first spreading them on the upper surface of solid 2% agar in sea water contained in a petri dish. This was essential to ensure uniform irradiation. In separate experiments, it was made sure that placing the eggs on agar did not have any effect on division. The eggs placed on agar divided at the same time as the controls in sea water.

a. Irradiation of Eggs

Unfertilized eggs were irradiated on agar, as described above, for 10 sec at the rate of 52 r/sec. These eggs were then fertilized with a dilute solution of nonirradiated sperm and allowed to develop at 15°C. Samples were taken every 5 or 10 min, fixed in Carnoy's solution (three parts absolute alcohol and one part glacial acetic acid), left in Carnoy's overnight; two changes of freshly made Carnoy's were made during a 24-h period. After another few hours, eggs were centrifuged and placed in 45% acetic acid. The samples could then be viewed under the phase microscope. Simultaneously, samples were also taken from nonirradiated controls, and all the samples were scored for interphases, early prophases, late prophases, prometaphases, metaphases, anaphases, telophases, and cleavages. The various phases are described in Appendix I. Early prophase corresponds, by definition, to the onset of chromosome condensation. All cells in and past this stage are scored as cells in which chromosomes have condensed.

Results of a typical experiment are listed in Table I, and curves based on data from this experiment are plotted in Fig. 1. The general features of the curves show that:

(a) Within 70 minutes after fertilization, the chromosomes in the controls start to condense, and 90 minutes after fertilization all the cells have condensed chromosomes.

(b) The chromosomes in the irradiated eggs do not show any condensation until later. If the 50% level is taken as the time of condensation, then, it can be seen that the chromosomes in the irradiated cells condense with a delay of 15-1/2 minutes.

(c) Similarly, by taking the cleavage time as the time when 50% of the cells have cleaved, it is seen that the irradiated samples cleave with a delay of 16-1/2 minutes.

From here on the same method is used to determine delay in condensation and division. Table II represents these delays computed for the five experiments.

Table I. Results of a typical experiment – unfertilized eggs irradiated with 520 r.

Time since fertilization (min)	I		EP		LP		PM		M		A		T		C	
	Co	Irr	Co	Irr	Co	Irr	Co	Irr	Co	Irr	Co	Irr	Co	Irr	Co	Irr
60	100	100														
65	100	100														
70	100	100														
75	88	100	12													
80	34	100	42		24											
85	6	96	20	4	30		43		1							
90		88	5	12	6		84		5							
95		66		30		4	59		41							
97		18		68		12		2								
100		2		52		40	24	6	67		9		0			
105		2		14		71	4	7	27	6	68		1			
110				3		55		12	10	30	84		6		0	
115						10		18	4	70	36	2	50		10	
120								3		53	4	44	24		72	
125										14		84	1	2	99	0
131										4		38		52		6
135												8		52		40
140														20		80
145																100

Co - Control samples
Irr - Irradiated samples
I - Interphase
EP - Early prophase
LP - Late prophase
PM - Prometaphase
M - Mataphase
A - Anaphase
T - Telophase
C - Cleavage

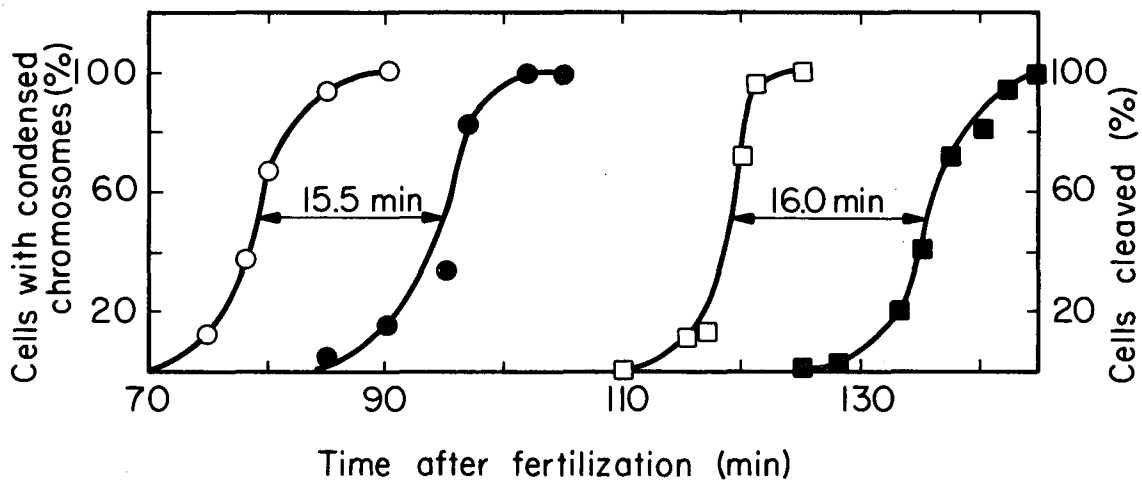

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Fig. 1. Irradiation of unfertilized eggs (dose 520 r).
○ Chromosome condensation in controls
● Chromosome condensation in irradiated eggs
□ Cleavage in controls
■ Cleavage in irradiated samples

Table II. Irradiation of eggs.

No.	Dose (roentgens)	Delay in chromosome condensation (min)	Delay in division (min)
1	520	15.5	16.5
2	780	16.0	14.0
3	1040	24.5	22.0
4	1040	21.0	25.0
5	1560	24.0	29.0

b. Irradiation of Sperm

Sperm were irradiated on agar plates for 10 sec at the rate of 52 r/sec. These irradiated sperm were used to fertilize nonirradiated eggs and the development was followed as for irradiated eggs.

Results of a typical experiment are plotted in Fig. 2. These curves show the following features:

(a) The chromosomes in the eggs fertilized by irradiated sperm condense with a delay of 14 min.

(b) The cleavage of the eggs fertilized by irradiated sperm occurs with a delay of 12-1/2 min.

Table III represents the delays computed for these five experiments.

Table III. Irradiation of sperm.

No.	Dose (roentgens)	Delay in chromosome condensation (min)	Delay in division (min)
1	520	14.0	12.5
2	780	17.5	16.0
3	1040	18.0	21.0
4	1040	22.0	21.0
5	1560	30.0	29.0

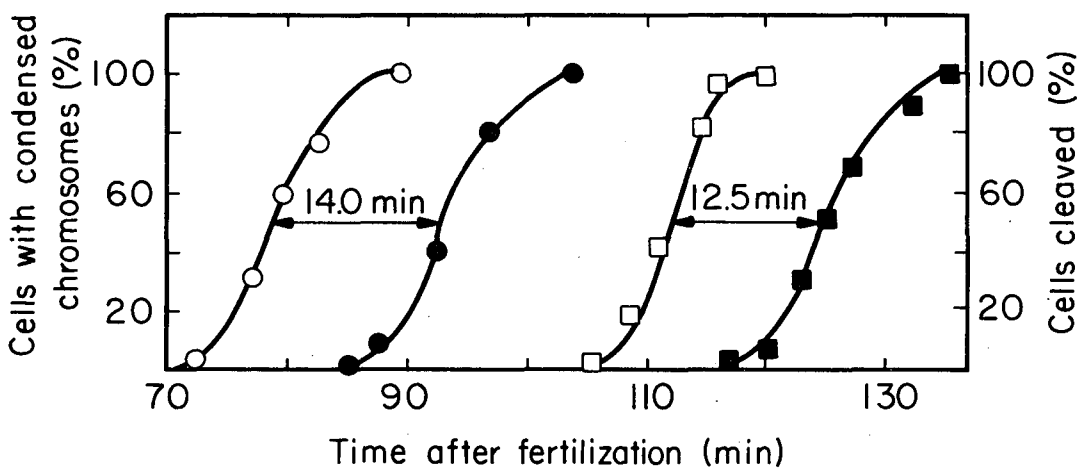

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Fig. 2. Irradiation of sperm (dose 520 r).
○ Chromosome condensation in controls
● Chromosome condensation in the irradiated samples
□ Cleavage in controls
■ Cleavage in the irradiated samples

c. Irradiation of the Zygotes

In these experiments, the eggs were fertilized and then irradiated at various times after fertilization. The dose in each case was 52 r/sec for 10 seconds. Samples were fixed every 5 to 10 min, as before. In one experiment, the development was followed through second cleavage to study the effect, if any, of irradiation on uncoiling. The results of these experiments are shown in Figs. 3, 4 and 5.

From Fig. 3 it is clear that eggs irradiated 70 min after fertilization show a delay of 24 min in chromosome condensation and a delay of 23-1/2 min in cleavage.

Figure 4 shows the results of an experiment in which the zygotes were removed 60 min after fertilization and irradiated. This time corresponds to the period when the cells are just entering prophase, which is the beginning of chromosome condensation. At 65 min after fertilization, 80% of the cells in the irradiated sample are in early prophase. Although most of the cells are arrested in early prophase and interphase, a few have passed the critical point and progress towards cleavage normally. These cells enter late prophase at the same time as the controls, but there is some delay before the rest of the cells enter late prophase. A similar pattern is followed by the cells during cleavage.

Figure 5 shows the results of an experiment in which the zygotes were irradiated 70 min after fertilization. There is a slight delay in the condensation of the chromosomes in the first division, but the main delay shows up in the second division. This delay in chromosome condensation corresponds to the delay in cleavage observed in the second division.

The duration of various phases of cell division in the control and the irradiated cells can be calculated as shown in Appendix II. The results are given in Table IV. It is evident that only interphase is prolonged in the irradiated samples. There is no appreciable delay in any other phase.

Table IV. Duration of various phases of cell division in control and irradiated zygotes.

Treatment of eggs	Interphase (min)	Early prophase (min)	Late prophase (min)	Metaphase (min)	Anaphase (min)	Telophase (min)
Non-irradiated	118	4	7.5	11	4	13
Irradiated	134	5	8	10	4.5	12.5

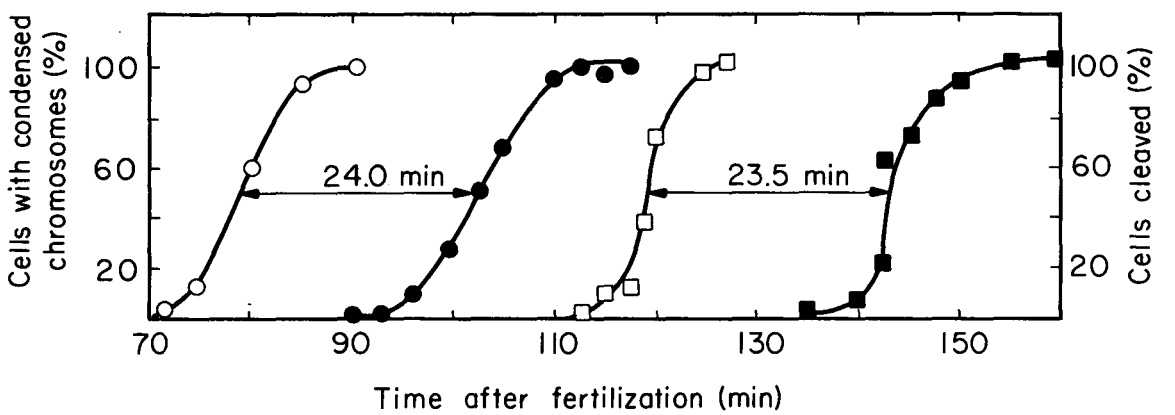

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Fig. 3. Irradiation of Zygotes 10 min after fertilization (dose 520 r).
○ Chromosome condensation in controls
● Chromosome condensation in the irradiated samples
□ Cleavage in controls
■ Cleavage in the irradiated samples

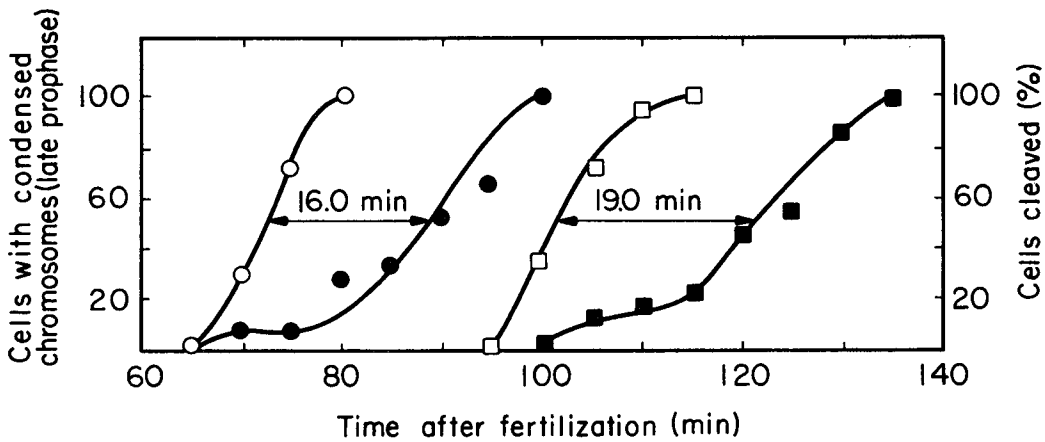

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Fig. 4. Zygotes irradiated just as the condensation of the chromosomes had started - 60 min after fertilization (dose 520 r).

○ Chromosome condensation in controls
● Chromosome condensation in irradiated samples
□ Cleavage in controls
■ Cleavage in irradiated samples

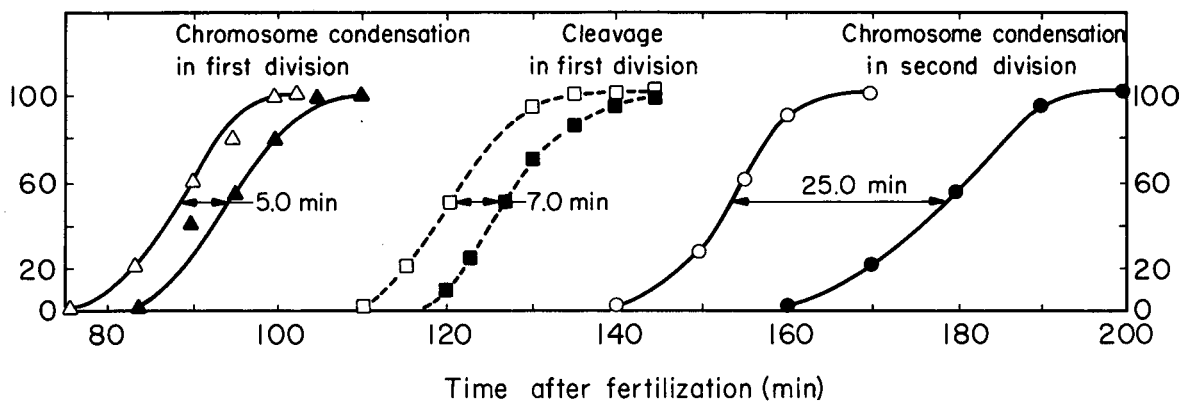

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Fig. 5. Irradiation of Zygotes 70 minutes after fertilization (dose 520 r).

△ Chromosome condensation in controls during first division

▲ Chromosome condensation in irradiated samples during first division

□ First cleavage in controls

■ First cleavage in irradiated samples

○ Chromosome condensation in controls during second division

● Chromosome condensation in irradiated samples during second division

2. Discussion

Experiments on irradiation of egg, sperm, and zygote demonstrate that in each case the onset of chromosome condensation is delayed. It is also seen that irradiation of the cells in early prophase--i.e., the time when chromosomes can be first resolved as microscopical threads--arrests the further condensation of these chromosomes for the time equal to the time of division delay. Once the cells pass through this block in early prophase, they go through the later phases at the same rate as the controls. Irradiation during late prophase or later stages induces delay of chromosome condensation in the next division. Carlson's (1954) results on living Chortophage neuroblasts are in general agreement with our observations, except that he finds that when higher doses of x rays are used to irradiate the cells in late prophase, the cells revert to a stage in which the chromatin resembles that of interphase. At lower doses the critical point (the point after which the division is insensitive to the effect of x rays) is reached at the end of prophase contraction about 5 minutes before the nuclear membrane breaks down.

In Figs. 6 and 7 it can be seen that aster formation and spindle formation are the same in irradiated samples and in controls. The only visible difference between the irradiated and nonirradiated eggs is that in the former the chromosomes have not condensed; there was no delay in the process of pronuclear fusion. These experimental results indicate very strongly that the antimitotic action of the x rays takes place through the channel of chromosome condensation.

It is not surprising that we know almost nothing about the process of chromosome condensation since so little is known about the molecular organization of the chromosome itself. Taylor (1963) has attempted to put forward three different molecular models for the chromosome. Similar attempts have also been made by other workers (Steffensen, 1959). Chromosome "condensation" is generally looked upon as the progressive coiling of the primary genetic thread (Kaufmann, 1948; Swanson, 1957). Anderson (1956) suggested a mechanism of chromosome coiling based on the interaction of nucleohistone threads, and predicted that polycationic compounds should cause chromosome coiling. Steffensen (1959) suggested that divalent cations have a stabilizing effect on chromosomes, probably by reducing the repulsion forces between negative phosphate groups. Mazia (1954), on the other hand, postulated that the chromosome is composed of complex macromolecules of DNA and protein held together by bridges of divalent cations. Thus it was possible to disassemble the chromosomes by treating them with a chelating agent capable of binding Ca, Mg^{++}, or both, and exposing them to a medium of low ionic strength.

A number of reports in the literature describe experiments in which chromatin condensation in the bacterial cells (Whitfield and Murphy, 1956) and in grasshopper neuroblasts (Hollaender, 1956) can be brought about by elevated salt concentrations and by polyamines (Anderson, 1960). At the molecular level Kaiser et al. (1963) demonstrate the stabilizing effect of polyamines on λ-phage DNA. They further show that the protection of DNA at a given concentration is a function of the amount of spermine present. These authors suggest that spermine may act by linking several DNA molecules together, thus simulating DNA concentration. Polyamines can form intermolecular complexes at high concentrations and intramolecular complexes

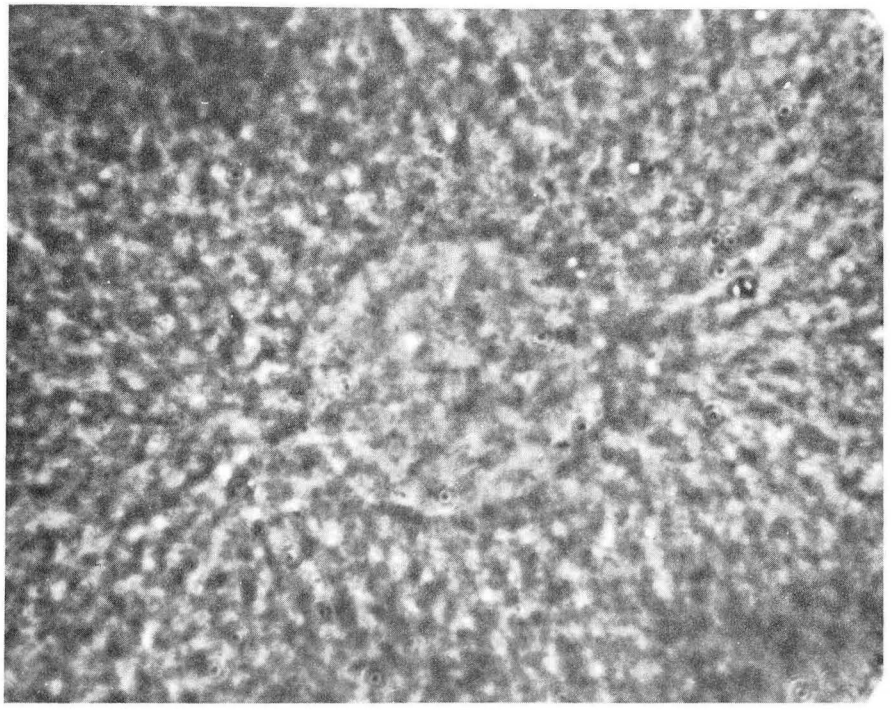

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Fig. 6. Aster and spindle formation in the irradiated eggs 90 min after fertilization (magnification 450 ×).

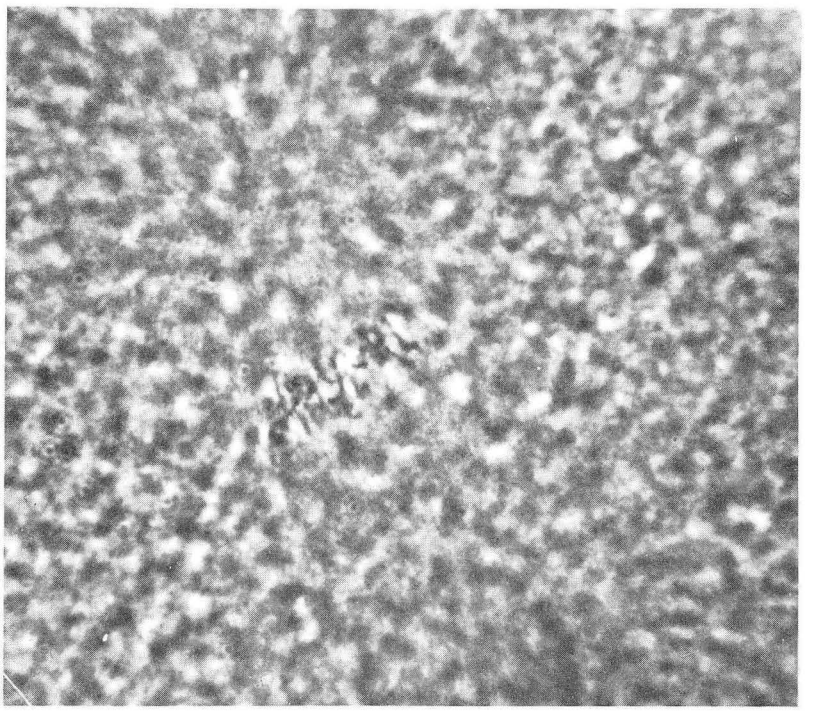

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Fig. 7. Aster and spindle formation in the non-irradiated eggs 90 min after fertilization (magnification 450 ×).

at low concentrations (Alexander, 1953). The fact that the condensation of DNA into precursor phage is abolished by chloramphenicol implies the existence of a chemical substance enabling the condensation process to occur. Kellenberger (1961) calls this the "Condensation Principle."

The only other chemical event known to be taking place at the time of chromosome condensation is the accumulation of RNA in the prophase chromosomes and shedding of this RNA during anaphase or telophase. This is known as the chromosomal RNA cycle and was first pointed out by Kaufmann et al. (1948). Jacobson and Webb (1952) and Boss (1954) have found the same cycle in animal cells. It is difficult at this stage to say whether chromosomal RNA variation is in any way related to chromosome condensation, but it seems to be a fruitful area for further investigations.

Evidence in favor of the relationship of chromosome condensation to the x-ray-induced division delay has come from the experiments of Whitfield et al. (1962). They have demonstrated the prevention of x-ray-induced mitotic delay in L-mouse cells by exposing the cells to agmatine before irradiation. It is known that agmatine causes a reversible condensation of chromatin and thereby in some way might counteract the effect of x rays on the condensation process.

B. Mercaptoethanol Experiments

Experiments with mercaptoethanol were done mainly to show further that x rays cause cleavage delay in these eggs by interfering with the process of chromosome condensation.

1. Experimental Design

Blocking of division by mercaptoethanol has been described in detail (Mazia, 1958; Mazia and Zimmerman, 1958). No attempt will be made to review this work here, but the following points should be noted:

a. In sea urchin eggs, the division could be blocked if mercaptoethanol were introduced at any time before a "point of no return," which has been located early in metaphase.

b. The blockage is fully reversible and the delay in division was exactly equal to the time spent in mercaptoethanol.

c. If the eggs were blocked just at metaphase, and were removed from the block at the time when the controls were in their second division, they would divide directly from one cell to four cells.

d. The effective concentration of mercaptoethanol, for blocking sea urchin eggs, lies in the range 0.075 to 0.1M.

The experiment described herein was based on the observation that there exists in telophase "a point of no return" (Mazia, 1960). If cells are placed in mercaptoethanol before this point, the cells go through the first division, but the condensation of the chromosomes in the second-division cycle does not take place so long as the cells remain in mercaptoethanol. On the other hand, if the cells are placed in mercaptoethanol after this point, the cells complete the first division and the chromosome condensation in the second division is unhindered. Thus, we have a situation in which we can obtain

two types of cells which are presumably similar in every aspect except the state of condensation of their chromosomes. It would be interesting to study the difference in the radiosensitivity of these two batches of eggs. The β-mercaptoethanol is being used here only as an analytical tool. The mechanism of the action of mercaptoethanol does not enter into the discussion of the results.

Eggs were fertilized as usual and allowed to develop in sea water at 15°C. At 100, 110, and 120 min after fertilization, samples of zygotes were removed and placed in 0.08 M β-mercaptoethanol soln in sea water. After the non-mercaptoethanol-treated eggs had gone through the second division, the eggs that had been placed in β-mercaptoethanol were washed three times with sea water. Half of the eggs from each sample were irradiated for 20 sec at 52 r/sec. The delay in division was evaluated in each case. The steps in these experiments are shown in Fig. 8.

The β-mercaptoethanol ($HS-CH_2-CH_2-OH$) was obtained from Eastman Organic Chemicals, Rochester, N. Y. Fresh solutions were made up every time.

The results of the experiment are shown in Figs. 9 and 10. The stage of development of cells just before irradiation, in samples A and B, is shown in Figs. 11 and 12. The following conclusions can be drawn:

a. In samples B and C, in which the chromosomes are condensed before irradiation, the irradiation does not cause any delay in cleavage.

b. Eggs in sample A show a delay in cleavage of about 16 min. In these eggs, the chromosomes were in an uncondensed state before irradiation.

2. Discussion

Bucher and Mazia (1960), using sea urchin eggs, have shown that mercaptoethanol does not inhibit the synthesis of DNA, and that DNA synthesis is independent of the duplication of the centers. It is also known that β-mercaptoethanol blocks the duplication of centers, but not their separation (Mazia et al., 1960). Mazia (1958) has also shown that mercaptoethanol, if applied before metaphase, blocks the formation of the mitotic apparatus. The stage of final arrest of development varies depending upon the stage at which the eggs are placed in mercaptoethanol. Apart from this information, very little is known about the effect of mercaptoethanol on other processes necessary for cell division. It is not unreasonable to believe that, if cells were left in mercaptoethanol long enough, some of the preparations for cell division (e.g., DNA synthesis) would proceed to completion, while others would be blocked. In our case, all three batches of cells, A, B, and C, were left in β-mercaptoethanol for 80 min, the time required for the controls to go through the second division. Thus, it is most probable that many of the prerequisites for division had been completed and that the cells had advanced to a critical stage and were then blocked. Cells placed in mercaptoethanol 100 min after fertilization were blocked in such a way that chromosome condensation could not take place. On the other hand, in those cells treated with mercaptoethanol 110 and 120 min after fertilization, chromosome condensation did occur.

The finding that irradiation of mercaptoethanol-treated cells in which the chromosomes have not condensed induces a delay in division supports the conclusion that the x rays cause this delay by interfering with the process of chromosome condensation.

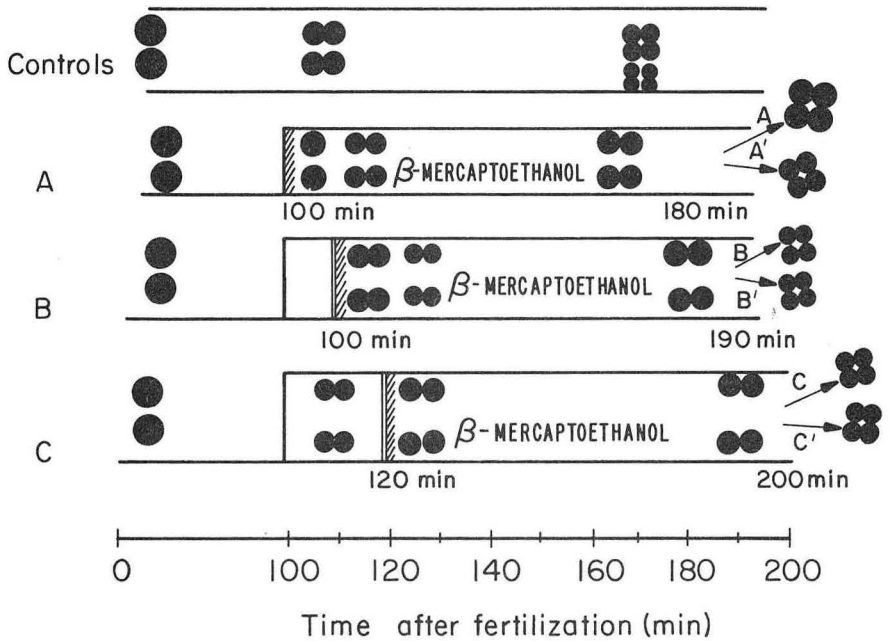

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Fig. 8. Design of β-mercaptoethanol experiments. Cells removed from sea water 100, 110, and 120 min after fertilization and placed in mercaptoethanol soln until controls have divided. Samples A', B', C' irradiated for 20 sec at 52 r/sec at indicated times.

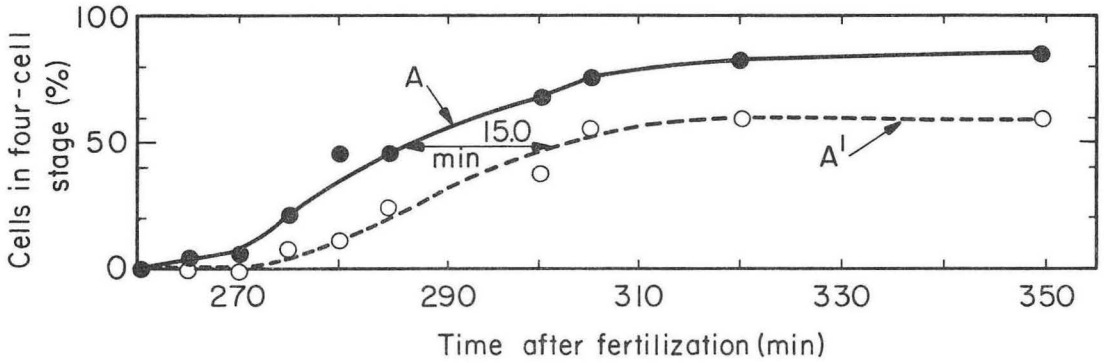

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Fig. 9. Effect of x rays on mercaptoethanol-treated cells.
Cells placed in mercaptoethanol 100 minutes after fertilization and transferred to normal sea water 180 minutes after fertilization. Chromosomes in these cells have not condensed while the cells were in mercaptoethanol.

A ● Controls
A' ○ Irradiated samples

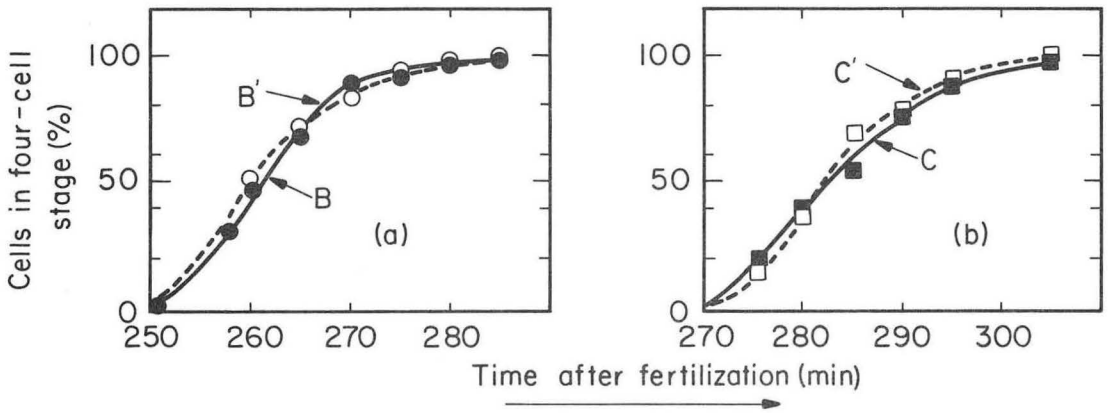

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Fig. 10. Effect of x rays on mercaptoethanol-treated cells.

(a) Cells placed in mercaptoethanol 110 minutes after fertilization and transferred to normal sea water 190 minutes after fertilization. In this time chromosome condensation in the cells has taken place.

B ● Controls
B' ○ Irradiated samples

(b) Cells placed in mercaptoethanol 120 minutes after fertilization and transferred to normal sea water 200 minutes after fertilization. In this time chromosome condensation in the cells has taken place.

C ■ Controls
C' □ Irradiated samples

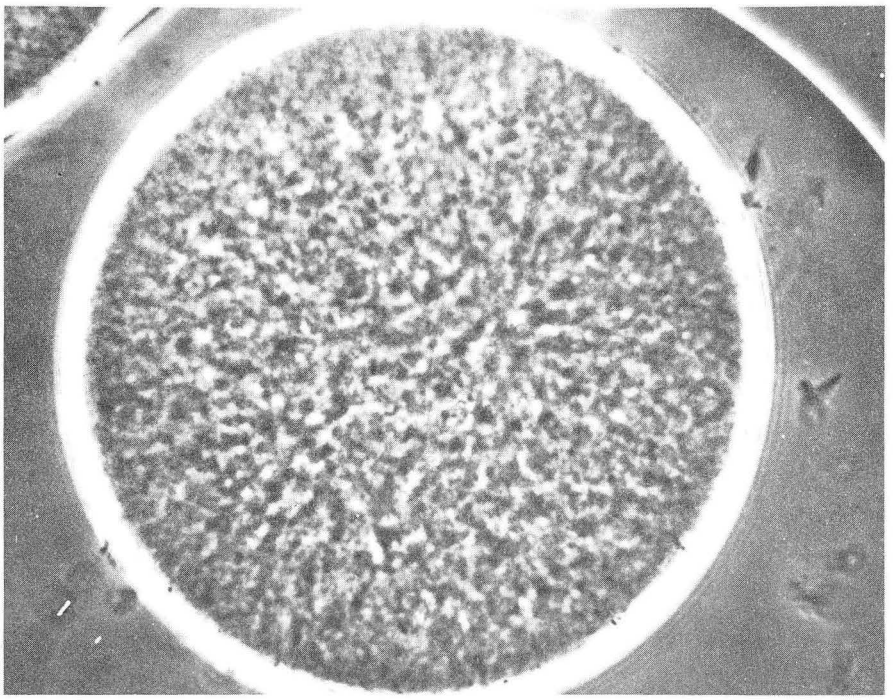

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Fig. 11. Mercaptoethanol samples with uncondensed chromosomes before irradiation (magnification 450 ×).

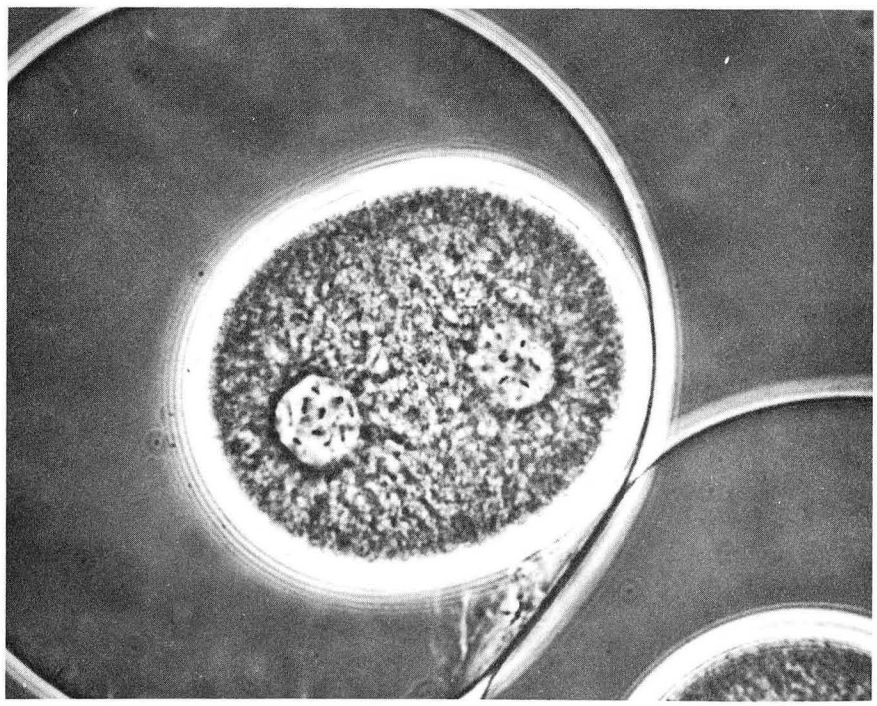

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Fig. 12. Mercaptoethanol samples with condensed chromosomes before irradiation.

C. Colcemide Experiments

Much discussion has been devoted to the problem of the site of the primary lesion produced by x rays. Experiments on irradiation of localized parts of cells have been carried out with widely different biological material. The results are often contradictory. No attempt will be made here to review the literature. An excellent review of the subject is given by Bacq and Alexander (1961).

Henshaw (1940) and Henshaw and Cohen (1940), working on Arbacia eggs, have shown that cleavage delay caused by x irradiation of either egg or sperm is the same. This is interpreted as indicating that x rays act mainly on the nucleus. Furthermore, if nucleated and nonnucleated egg fragments obtained by centrifugation are irradiated and then fertilized with nonirradiated sperm, the delay occurred in the nucleated fragments, whereas no delay occurred in the nonnucleated fragments (Henshaw, 1938). The time required for division of the nonnucleated eggs is abnormally long. On the other hand α-ray experiments performed by Miwa, Yamashita, and Mori (1939) indicated that eggs showed a marked delay in division even with α-ray ranges that fell short of the nucleus.

Ulrich (1955) took advantage of the fact that in unfertilized eggs of Drosophila the nucleus always lies in the anterior part of the egg. It was thus possible to irradiate either the nucleus or the cytoplasm. Using hatchability as the criterion of radiosensitivity, he found that the nucleus was 185 times as sensitive to x rays as the cytoplasm.

Alpha irradiation of chromosomal regions of chick fibroblasts in tissue culture produced sticky bridges at anaphase. When the cytoplasm was irradiated with 30 times the dose, no abnormalities were seen (Munro, 1957).

Duryee (1949) carried out remarkable investigations on amphibian eggs--convenient material for microsurgery. He found that nuclei freed of cytoplasm are very radioresistant. Doses as high as 30,000r had very little effect. If isolated nuclei were replaced in the cytoplasm and the whole reconstituted egg were irradiated, the nucleus became very radiosensitive. If the nucleus of an irradiated egg was transferred into the enucleated cytoplasm of a normal cell, no lesions were seen.

Thus it becomes clear that the results from experiments done on insect egg, amphibian eggs, and tissue culture cells are somewhat contradictory.

In many of these experiments it is difficult to irradiate the nucleus or the cytoplasm without affecting the other. Usually, comparison is made between irradiating (a) a certain portion of the cytoplasm, and (b) the nucleus together with adjacent cytoplasm. Also, since doses used to irradiate the cells are very high, most of the cells suffer irreversible damage, which is difficult to evaluate.

In our work we were concerned mainly with the mechanism underlying reversible mitotic delay. The experiments described in the preceding two sections led to the conclusions that reversible mitotic delay, caused by x rays, was a result of interference in the chromosome condensation process. Is the

condensation process of purely nuclear origin, or does the cytoplasm contribute to it? Colcemide experiments were done mainly to elucidate this question.

1. Experimental Design

It is known that colchicine, which blocks the operation of the spindle, does not stop the condensation of the chromosomes (Eigsti and Dustin, 1955). The nuclear membrane also breaks down, but chromosome movement is prevented. It was found that if sea urchin eggs were placed in a 0.01% soln of colcemide (a derivative of colchicine) just after fertilization, the fusion of sperm and egg nuclei was blocked (Sauaia, 1959). Chromosome condensation and breakdown of the nuclear membrane proceeded normally in the two nuclei. It is thus possible to irradiate either the sperm or the egg, separately, and to study the effect on chromosome condensation in the two nuclei. It is important to stress that colcemide is being used as an experimental tool, and its mode of action is not considered in the discussion of the results.

2. Irradiation of Sperm

Sperm were irradiated on agar for 30 sec at the rate of 52 r/sec. Half of the irradiated sperm was used to fertilize nonirradiated eggs, which were then placed in a 0.01% soln of colcemide. The rest of the irradiated sperm was used to fertilize another batch of nonirradiated eggs. At the same time two batches of nonirradiated eggs were fertilized with nonirradiated sperm. One of these fertilized batches was placed in 0.01% colcemide solution. This is the colcemide control. Thus, we had the following four batches of eggs (Fig. 13):

Nonirradiated sperm, non-colcemide control
Nonirradiated sperm, colcemide control
Irradiated sperm, non-colcemide eggs
Irradiated sperm, colcemide eggs

The eggs were allowed to develop at 15°C and, beginning at 60 min, samples were fixed every 5 or 10 min, as in the previous experiments. Samples were scored for condensed chromosomes in both the egg and the sperm nuclei.

Figures 13a and 13b present the results of this experiment. In Fig. 13a the ordinate represents the percent of colcemide-treated cells in which the chromosomes have condensed, whereas the abscissa refers to the time after fertilization. The four curves represent the egg and the sperm chromosomes in the irradiated and nonirradiated colcemide samples.

The following points are to be noted:

(a) Chromosomes in the sperm nuclei of the colcemide-treated cells condense with a delay of about 10 minutes compared with the chromosomes in the egg nuclei.

(b) Irradiation of the sperm does not affect the condensation of the chromosomes in the egg nucleus.

(c) There is a delay of about 20 min in the condensation of irradiated sperm chromosomes.

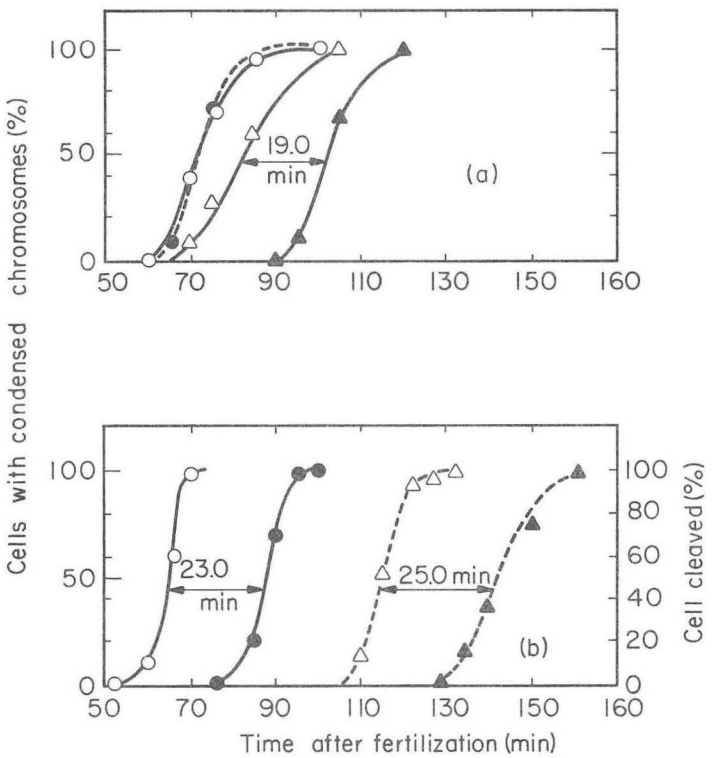

Fig. 13. Irradiation of sperm (dose 1560 r); (a) colcemide-treated eggs,

○ Egg chromosomes in nonirradiated samples, E_c

● Egg chromosomes in irradiated samples, E_r

△ Sperm chromosomes in nonirradiated samples, S_c

▲ Sperm chromosomes in irradiated samples, S_r

(b) non-colcemide-treated eggs.

○ Zygotes (fertilized with nonirradiated sperm) with condensed chromosomes (%), C

● Zygotes (fertilized with irradiated sperm) with condensed chromosomes (%), C_r

△ Zygotes (fertilized with nonirradiated cells) cleaved (%), C

▲ Zygotes (fertilized with irradiated cells) cleaved (%), C_r

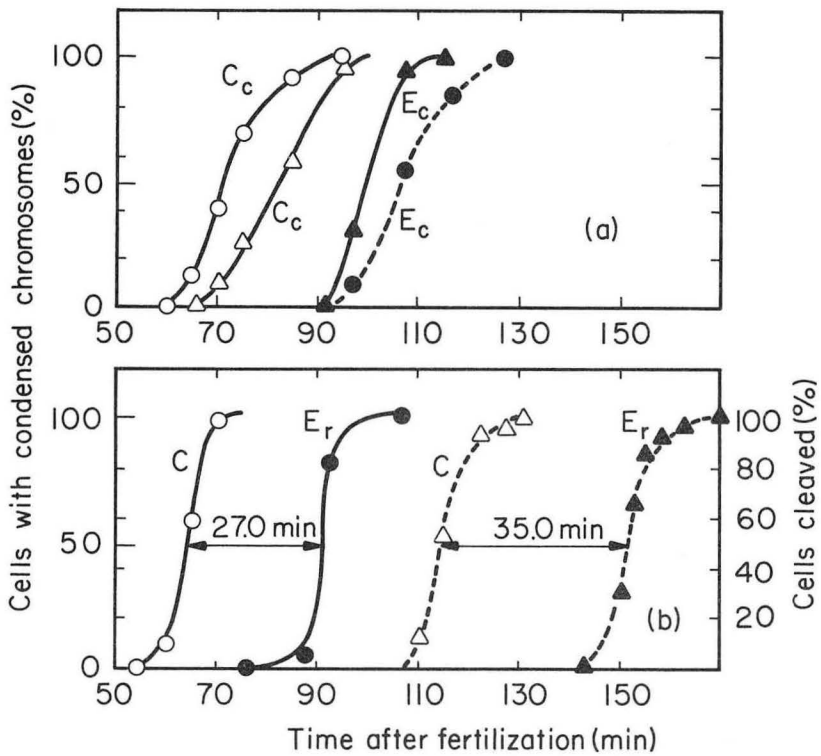

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Fig. 14. Irradiation of unfertilized eggs (dose 1560 r)

(a) Colcemide-treated eggs,

○ Egg chromosomes in nonirradiated samples E_c

● Egg chromosomes in irradiated samples E_r

△ Sperm chromosomes in nonirradiated samples S_c

▲ Sperm chromosomes in irradiated samples S_r

(b) non-colcemide-treated eggs.

○ Nonirradiated cells with condensed chromosomes (%) C

● Irradiated cells with condensed chromosomes (%) C_r

△ Nonirradiated cells cleaved (%) C

▲ Irradiated cells cleaved (%) C_r

(d) The delay in condensation of the sperm chromosomes is almost equal to the delay in condensation of the noncolcemide-irradiated eggs, which in turn corresponds to the delay in cleavage. The comparison is shown in Fig. 13b.

3. Irradiation of Eggs

The procedure was the same as that in the previous experiment except that, in this case, the eggs were irradiated instead of the sperm. The x-ray dose was 52 r/sec for 30 sec.

Results are presented in Figs. 14a and 14b, and show the following:

a. As in the preceding experiment, the sperm chromosomes in the colcemide controls condense with a delay of about 10 min.

b. Delay in the condensation of the irradiated egg chromosomes E_r is 34 min.

c. The condensation of the sperm chromosomes in the colcemide irradiated samples S_r is also delayed.

d. The delay in the chromosome condensation of the egg nuclei E_r corresponds to the delay in chromosome condensation and to the delay in cleavage found in irradiated non-colecemide-treated cells.

4. Irradiation of Both Gametes

In these experiments, the sperm and eggs were irradiated separately for 30 sec at 52 r/sec. The irradiated eggs were then fertilized by the irradiated sperm and placed in 0.01% solution of colcemide. The rest of the procedure was the same as in the two preceding experiments.

Figure 15(a) and Fig. 15(b) present the results of the experiment. The data on these figures show the following:

a. In the irradiated colcemide-treated cells, condensation of both the egg chromosomes and the sperm chromosomes is delayed by almost the same amount (Fig. 15a).

b. These delays in chromosome condensation are of the same order of magnitude as the delay in cleavage in non-colcemide-treated irradiated cells (Fig. 15b).

5. Discussion

Radiosensitivity of the nucleus is quite evident from the experiments in which either the egg or the sperm is irradiated. The fact that irradiation of the egg causes a delay in the condensation of the sperm chromosomes points very strongly to the role of the cytoplasm in radiation-induced injury. It could perhaps be argued that the primary effect of x rays is only on the egg nucleus, which in some way exerts an influence on the sperm nucleus. If this were so, then the irradiation of the sperm should have similarly influenced the chromosome-condensation process in the egg nucleus. This, we found, was not true. Daniels's (1952, 1958) work on amoeba also points to the importance, in radiation injury, of both the nucleus and the cytoplasm. In general, Duryes's work on amphibians' eggs (1949), Daniels's experiments on amoeba (1958), Hammerling and Brachet's work on Acetabularia, and our work with the sea urchin eggs all lead to the following common points:

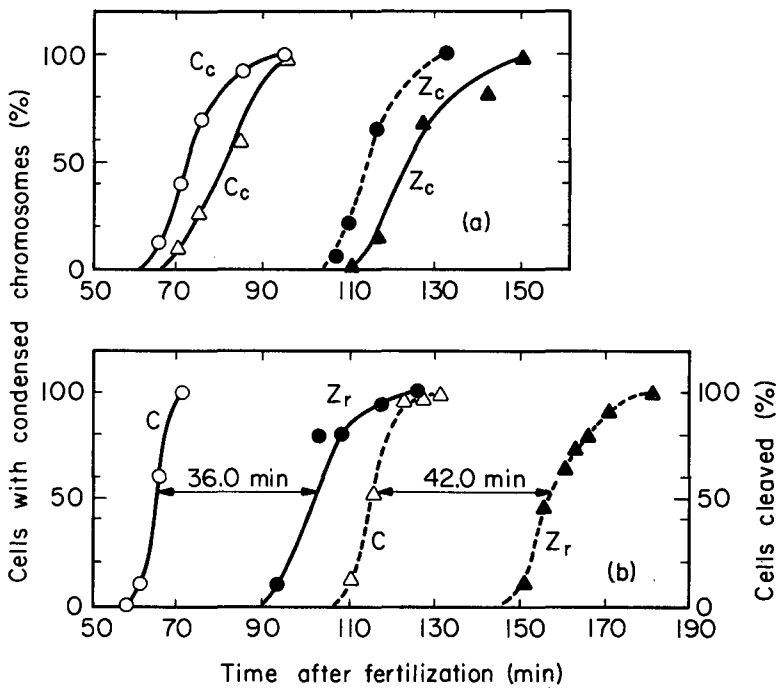

Fig. 15. Irradiation of both gametes; (a) colcemide-treated eggs, dose in each case 1560 r

○ Egg chromosomes in nonirradiated samples E_c

● Egg chromosomes in irradiated samples E_r

△ Sperm chromosomes in nonirradiated samples S_c

▲ Sperm chromosomes in irradiated samples S_r

(b) non-colcemide-treated eggs. Dose in each case 1560 r

○ Nonirradiated cells with condensed chromosomes (%), C

● Irradiated cells with condensed chromosomes (%), C_r

△ Nonirradiated cells cleaved (%) C

▲ Irradiated cells cleaved (%) C_r

(a) Both the nucleus and the cytoplasm are radiosensitive.

(b) Irradiated cytoplasm has the power to cause injury to the nonirradiated nucleus, whereas the normal cytoplasm has the capacity to repair the radiation injury.

If we accept the hypothesis that x-ray-induced mitotic delay is the result of the disturbance of the chromosome-condensation process, then it is quite clear that sites of primary lesion are in both the nucleus and the cytoplasm. Although radiation damage ultimately shows up in a chromosomal process, chromosomes are not the only sites of the primary lesion. It seems more reasonable to consider the phenomenon of reversible mitotic delay as a disturbance in some metabolic processes of the cell, rather than a damage to some particular structure in the cell. The latter hypothesis has been put forward by Sparrow (1950) and Puck (1961). According to these authors, reversible mitotic delay may represent an action basically similar to that underlying irreversible killing by ionizing radiations.

Bacq and Alexander (1961) propose the hypothesis of enzyme release, according to which the primary lesion for cell death due to irradiation is an alteration in the permeability of certain intracellular structures. This, in turn, releases certain enzymes that are harmful to the cell. The same hypothesis could be extended to explain reversible mitotic delay. As Bacq and Alexander (1961) point out in their book: "The controversy as to whether the nucleus or the cytoplasm is the radiosensitive part of the cell, loses meaning, if there is an enzyme release mechanism since it is possible for cytoplasmic enzymes to act both on cytoplasmic structures and on nuclear structures, since the nuclear membrane allows large molecules to permeate." The release of the harmful substances could cause the destruction of some substrate in the cell that is necessary for chromosome condensation. Unfortunately, there is very little evidence that enzymes are set free by doses as small as those used in our study. Hagen (Bacq, 1961) has found some support for the view that certain proteolytic enzymes are released from mitochondria by doses of less than 1000 r. Our experiments with colcemide do not prove or disprove the enzyme release hypothesis, but, in an indirect way, lend support to it.

III. THE EFFECT OF LOW DOSES OF X RAYS ON DNA SYNTHESIS

Since the classical experiments of Euler and Hevesy (1942), in which they showed that x rays decreased the incorporation of P^{32}-orthophosphate into DNA of Jensen sarcoma, a voluminous literature on the effects of x rays on DNA synthesis has accumulated. No attempt will be made to review the literature completely. For recent reviews, Kelly (1957, 1961), Lajtha (1960), and Stocken (1959) should be consulted.

Suppression of DNA synthesis by x irradiation has been shown in many instances, and much work has been done to determine how the radiosensitivity of DNA synthesis varies during the mitotic cycle (Howard and Pelc, 1953; Beltz et al., 1957; Lajtha et al., 1958; Sherman and Quastler, 1960; Paul, 1960; Painter, 1962). It has been observed, in general, that relatively low doses of x rays delivered to cells in the G_1 period interfere with the ability of these cells to enter DNA synthesis. Higher doses, however, are required to diminish the rate of DNA synthesis once it has started. Bollum et al. (1960) suggest that irradiation in the G_1 period blocks synthesis of enzymes required for DNA duplication, whereas irradiation during the S period damages the priming ability.

Das and Alfert (1961), working with onion root meristem, have demonstrated that not only does DNA synthesis continue after irradiation, but that synthesis is also actually stimulated during irradiation. Yamada and Puck (1961) have shown that x irradiation of S_3 Hela cells has no affect on their DNA synthesis.

Although there is no doubt that high doses of x rays affect DNA synthesis, the question remains whether reversible mitotic inhibition, produced by low doses of x rays, is the result of interference with DNA synthesis. Two opposing views have been advanced:

a. Mitotic delay is due to the inhibition of DNA synthesis, the latter being the primary effect of ionizing radiations (Errera, 1957; Cardella and Servello, 1960).

b. Reduced DNA synthesis is a result of radiation-induced mitotic delay. This view was first put forward by Kelly (1957), and later confirmed by other workers (Whitfield and Rixon, 1959; Caspersson et al., 1958; Whitmore et al., 1961; Painter and Robertson, 1959; Harrington, 1960).

It is difficult to evaluate the results of experiments performed on asynchronous cell populations. More precise information about the effect of x rays on different biochemical processes and their relation to division delay can be obtained by using synchronous cell populations. Sea urchin eggs provide an ideal experimental material. In this chapter, experiments are described that deal with the effect of x rays on DNA synthesis and its relationship to reversible mitotic delay.

A. Materials and Methods

Eggs and sperm from the sea urchin *Strongylocentrotus purpuratus* were obtained by the methods already described. Unfertilized eggs were irradiated with x rays for 30 sec at a dose rate of 52 r/sec. After irradiation these eggs were placed in sea water containing H^3-labeled thymidine at a

concentration of 5 μC/ml (specific activity, 6.7 C/mM). At the same time a sample of nonirradiated eggs was also placed in H^3-labeled thymidine. The concentration of eggs in each of these samples was 2×10^4 eggs/ml. Both samples of eggs were fertilized and allowed to develop at 15°C. Two samples were taken simultaneously every 5 minutes from each of these batches--one for liquid-scintillation counting, and the other to determine the stage of development of the eggs at each of these points. The eggs were fixed in Carnoy's solution (described in Chapter II) and viewed under the phase microscope. Samples for scintillation counting were prepared as follows. A 2-cc sample of egg suspension was placed in 6 cc of Carnoy's and left overnight. The eggs were then centrifuged at 1000 rpm for 1 min and placed for 20 min in 3 cc of 1 M perchloric acid at room temperature. After another centrifugation, the eggs were washed with 3 cc of 100% methanol. After centrifuging again, the methanol was discarded, and 1 cc of hyamine was added to the eggs. It was necessary to heat this slightly in order to dissolve the eggs completely. This final solution of hyamine was added to 10 cc of liquid-scintillation solution and counted.

B. Results

The results of the experiment are shown in Fig. 16. Here the dotted line represents the incorporation of H^3-thymidine into the DNA of nonirradiated eggs during two division cycles, and the solid line represents incorporation into irradiated eggs. The curves at the bottom of the figure represent the time and rate at which the eggs go through division.

C. Determination of Time and Period of DNA Synthesis

Many workers, using photometric and autoradiographic techniques, have determined the time and period of DNA synthesis in various cells (Swift, 1950; Howard and Pelc, 1953; Lajtha et al., 1954; Taylor and McMaster, 1954; Painter and Drew, 1959; Stanners and Till, 1960; Puck, 1961). In general, the procedure has been to determine the grain count over metaphases at increasing times after addition of the label. The grain counts should remain zero until the end of the G_2 period, after which they should continue to increase for the time equal to the period of DNA synthesis, and then become constant. The autoradiographic method for determining the S period is, in general, quite laborious, and errors are introduced owing to a number of variables.

In the case of sea urchin eggs, which have a high degree of synchrony, an expression can be derived to determine the amount of incorporation of labeled thymidine into the DNA of these eggs at any given time.

Let N(t) be the number of cells that have begun incorporating labeled thymidine by time t; N(t) is a function of t and is of the form shown in Fig. 17a. The shape of this curve can be determined experimentally by plotting percent cells, in and past any of the division stages, against time (Table I).

The label is added at time $t = 0$ (Fig. 17b), which is the time at which the cells are fertilized. Let $t = t_0$ be the time at which the total number of counts observed is $C(t_0)$. Also let S be the period of DNA synthesis for an individual cell. It is assumed that S is the same for all the cells.

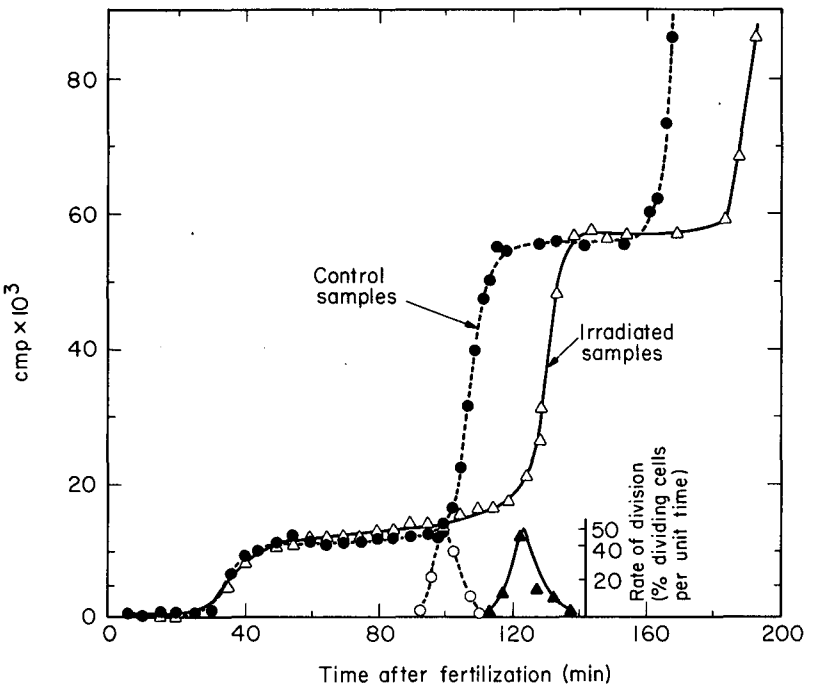

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Fig. 16. Effect of x rays on DNA synthesis in sea urchin eggs.

H^3 - thymidine incorporated into DNA of standard aliquot of egg suspension during two division cycles.

Eggs irradiated before fertilization for 30 sec at the rate of 52 r/sec.

○ Nonirradiated eggs

△ Irradiated eggs

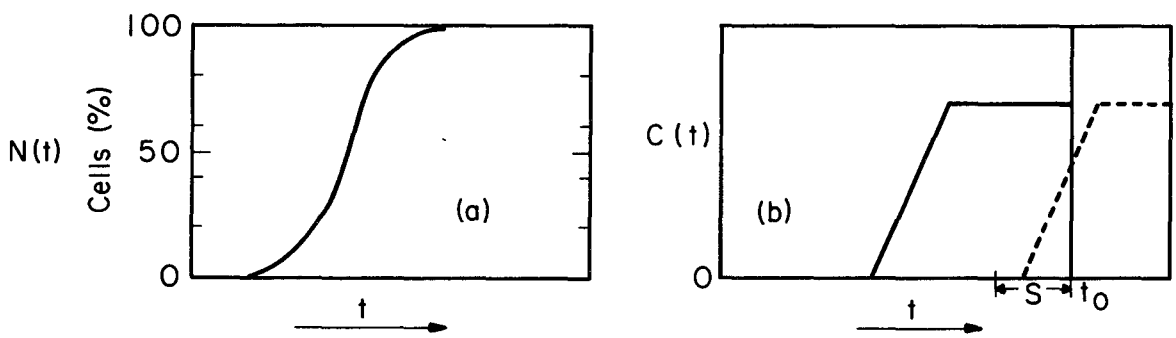

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Fig. 17. (a) N(t) = percentage of cells that have begun incorporating thymidine by time t. (b) total number of counts as a function of time.

The cells that have begun incorporating labeled thymidine by time t can be divided into two groups, those which start incorporation of labeled thymidine before time $(t_0 - S)$ and those which start after time $(t_0 - S)$.

The contribution to $C(t_0)$ of the first group of cells is equal to $N(t_0 - S)\, K \cdot S$. K is the rate constant for incorporation of labeled thymidine.

Now consider those cells which start incorporating the label after the time $(t_0 - S)$.

The number of cells that begin incorporating the label in time dt is $(dN(t)/dt) \cdot dt$.

Therefore, we now obtain $dC = \frac{dN(t)}{dt} \cdot dt \cdot K(t_0 - t)$.

Hence, the contribution to $C(t_0)$ of all the cells which begin synthesis between t_0 and $(t_0 - S)$ is

$$\int_{t_0 - S}^{t_0} \frac{dN(t)}{dt} \cdot dt \cdot K(t_0 - t) .$$

Therefore we find

$$C(t_0) = N(t_0 - S) \cdot K \cdot S + \int_{N(t_0 - S)}^{N(t_0)} K(t_0 - t)\, dN(t) .$$

The above equation can be simplified to yield

$$C(t_0) = K \int_{t_0 - S}^{t_0} N(t)\, dt ,$$

or

$$C(t) = K \int_{t - S}^{t} N(t)\, dt . \qquad (1)$$

If all the cells entered the DNA synthesis period at the same time, $N(t)$ would be constant, and then $C(t) = C_{max} = KNS$. In the case of a sea-urchin-egg population, $N(t)$ can be represented by the equation

$$N(t) = \frac{1}{\sigma\sqrt{2\pi}} \int_{-a}^{t} e^{-(t-t_1)^2/2\sigma^2}\, dt , \qquad (2)$$

which is the equation for normal distribution. Here t_1 is the mean and represents the standard distribution. When N(t) is determined experimentally by scoring cells in different stages of division, it fits the above equation.

Substituting this value of N(t) in Eq. (1), we obtain

$$C(t) = \frac{K}{\sigma\sqrt{2\pi}} \int_{t-S}^{t} \int_{-a}^{t} \exp[-(t-t_1)^2/2\sigma^2]\,dt \quad . \qquad (3)$$

In the experiment, C(t) was determined as a function of t and the parameters t_1 and S were determined by least-squares fits with the help of the 7090 computer.

Time and duration of DNA synthesis are shown in Table V.

Table V. Time and duration of DNA synthesis in the irradiated and nonirradiated eggs.

	Period of DNA synthesis (min)	Time at which DNA synthesis for second division begins (min after fertilization)
Irradiated eggs	11.5	127.6
Controls	10.7	102.5

D. Discussion

Figure 16 shows that DNA synthesis for the first division begins about 25 min after fertilization; i.e., after the sperm and the egg nuclei have fused. DNA synthesis in the irradiated eggs is not affected. The lag in the second division is due to the fact that the irradiated eggs divide with a delay of about 25 min. This, in turn, delays the synthesis of DNA by the same amount of time. Unlike many other cell types, DNA synthesis in the sea urchin eggs starts some time in late telophase, which corresponds to the time when chromosomes reach the poles and begin to uncoil.

The duration of DNA synthesis (S period) in both the irradiated and nonirradiated eggs is the same. Therefore, our experiments show not only that sublethal doses of x rays, which cause reversible mitotic delay, do not affect DNA synthesis, but also that the mitotic activity may in some way regulate DNA synthesis.

IV. DIVISION DELAY AS A FUNCTION OF DOSE AND TIME OF IRRADIATION

A number of studies both in viva and in vitro have described the phenomenon of x-ray-induced mitotic delay. Different aspects of the phenomenon have been studied in widely different materials, e.g., invertebrates' eggs, various plant and animal tissue, and bacteria. No attempt is made here to review all the literature on the subject. (For an excellent review, see Carlson, 1954; and Lea, 1947). Often the results are difficult to interpret because of the asynchrony in the cell population, and because of the unknown degree to which the reversible and irreversible damages have contributed to the effect. The practice generally is to determine the mitotic index as a function of time after irradiation, and to define as division delay the time required to reach some point on the curve, frequently the time at which the mitotic index reaches a minimum. Since the cells in a population are present at various stages, which differ in their relative radiosensitivity, it is very difficult to relate this gross value of division delay to any cellular event. The importance of considering the variation of radiosensitivity with different stages in the division cycle, in an asynchronous population, has become more evident from the findings of Terasima and Tolmach (1963). They synchronized Hela cells, irradiated the cells at different stages of the cycle, and found that radiosensitivity varied continuously.

The dependence of division delay on the dose and stage of irradiation is of considerable interest in interpreting the basic cellular effects of radiation. These studies may also point out the relationship of division delay to the lethal damage. A comparison of the responses of the two phenomena--division delay and lethality--to different kinds of radiations, to various doses, and to ploidy, should give some clues to the relationship, if any, among these processes.

Working on this principle, Gray (1950) provides a good illustration of the fundamental difference between lethality and division delay. His work with bean roots showed that the relative efficiencies of γ rays, neutrons, and x rays in causing the two effects were quite different. On the other hand, Burns (1954), working with yeast, has shown that a given dose of x radiation produces about five times as much delay in nonbudding cells as in budding cells; this behavior agrees qualitatively with that of lethal damage in these cells. He also showed that mean division delay is not strongly dependent on ploidy; this is in contrast to the observation on lethality.

In determining the relation between a dose of radiation and the biological changes induced, it is important to state clearly the type of effect being investigated and the criterion used to define it. Because of the randomness of the ionization produced, a wide variety of changes is caused by x rays at the cellular level. It is possible to study the phenomenon of reversible mitotic delay, if sufficiently low doses are used to prevent the occurrence of other irreversible changes. The criterion of reversible mitotic delay used in this work was developed as follows.

The delay showed up in only one generation, i.e., if the cells were irradiated before the critical point, the delay showed up in that division only and all the subsequent divisions were normal. However, if the cells were irradiated after the critical point, second division was delayed but there was no

delay in the division following the second. The development of the eggs was observed up to the hatching stage, which is reached 20 h after fertilization. Only those doses which allowed 100% hatching were employed. It was found that a dose of x rays greater than 2000 r interfered with the process of hatching of these eggs. Doses used by previous investigators (Henshaw, 1940; Yamashita et al., 1939), working with sea urchin eggs, were very large (on the order of 2600 r to 249, 600 r). In addition Henshaw (1940) extended his findings only to the first cleavage. The effect of such large doses on the subsequent division is not known. In our work, we found that at doses higher than 2000 r the divisions were very abnormal and many eggs did not go through a second division.

The work presented in this section is in many respects similar to that of Henshaw and his co-workers.

A. Variation of Division Delay with Dose of Irradiation

1. Materials and Methods

A suspension of eggs was fertilized, equally divided into seven parts, and placed on separate petri dishes containing 2% agar in sea water. One dish was not irradiated and was used as a control. The eggs in the other six dishes were allowed to develop on the agar for 40 minutes after which they were irradiated for 1, 5, 10, 20, 40, and 60 sec respectively (at the rate of 52 r/sec). After irradiation all the samples, including the control samples, were transferred back to 250-cc beakers containing sea water at 15°C. The maximum time of irradiation was 1 min, which, being a very small fraction of the total time of division, could not contribute any appreciable error in the results. Just before the start of cleavage, samples were taken every 2 or 5 min and placed in 2% formalin in sea water. Time of 50% cleavage was determined by viewing the formalin-fixed cells under the microscope and determining the percentage of cells cleaved as a function of time. A cell was counted as cleaved when the cleavage furrow started to appear. The time at which 50% of the cells were cleaved was taken as the time of cleavage and can be determined to an accuracy of about 1 min.

2. Results

Results of three experiments are shown in Fig. 18; the same results plotted on a log scale are shown in Fig. 19. These experiments showed that division delay increases with dose. The initial rate of increase is higher than the final rate, which after an initial high value becomes constant at larger doses. Although qualitatively the three experiments seem to agree quite well, the quantitative variation is considerable.

3. Discussion

An attempt was made to explain the quantitative variation of the three experiments on the basis of difference in generation times, the rationale behind this being that the cells with longer generation time might have a greater opportunity for repair of irradiation damage. Thus the division delay for a given dose in the cells with a longer generation time should be less than in those cells which have a shorter generation time. This was not found to be true. In fact, cells with a longer generation time (115 min) are more sensitive to radiation than those with a shorter generation time (98 min) (Fig. 18).

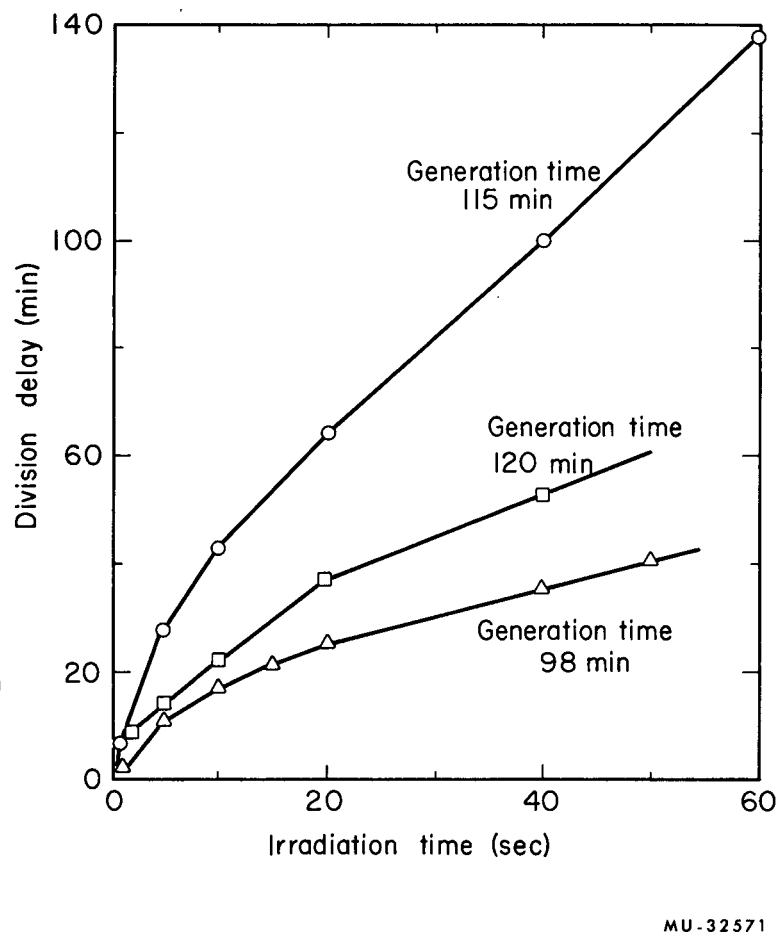


Fig. 18. Division delay as a function of dose of irradiation plotted on a normal scale (dose rate 52 r/sec).

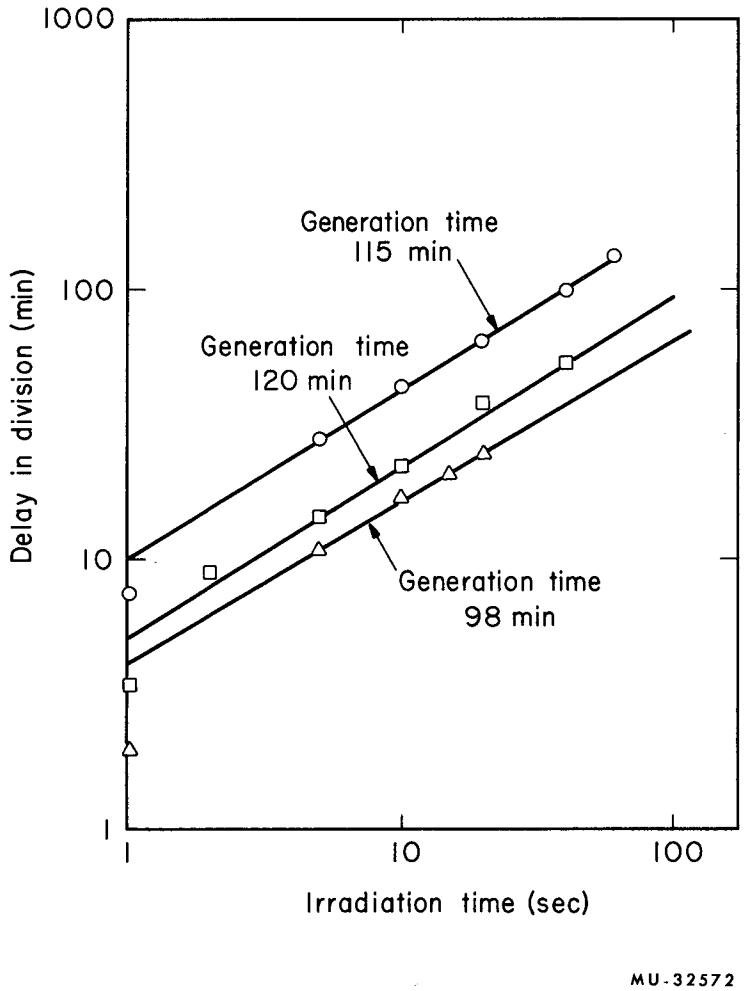


Fig. 19. Division delay as a function of dose of irradiation plotted on a log scale (dose rate 52 r/sec).

Before an attempt is made to explain the curves in Fig. 18, it will be useful to review some of the ideas about possible modes of biological action of radiations.

It is known that ionizing radiations, while traversing through matter, produce intense and localized releases of energy along their path. The energy released is sufficient to cause chemical alterations in the molecules. Setlow and Pollard(1962) have made some rough calculations to show that, even at high doses, the number of molecules altered by radiation is very small. Thus, one of the main problems in radiobiology is to explain how significant biological effects are produced by doses of radiation that produce negligible chemical effects. Various hypotheses have attempted to explain this phenomenon (Lea, 1946). Target theory is based on the idea that the biological effect observed is due to the production of ionization in some critical molecule or structure in the cell. It is possible to calculate the volume of the critical molecule or structure if one knows the D_{37} dose, i.e., the dose that corresponds to an average of one hit per target.

It is interesting to note that the theory originated from the attempt by Crowther (1924) to explain inhibition of cell division with dose. He showed that variation of inhibition of cell division with dose could be quantitatively explained if it were considered that this inhibition was the result of a single ionization in a volume which corresponded to the size of the centromere.

Lea, Haines, and Coulson (1937) have explained the killing of bacteria as a lethal mutation produced by a single ionization within a single volume that corresponds to the chromosomal volume. Similarly Puck (1956, 1958), working with tissue-culture cells, showed that his results can be explained in terms of target theory if the damage is considered to have occurred in one or more chromosomes. There seems to be no reason why the same target-theory type of explanation cannot be used to explain other types of radiation effects, such as division delay. Using the concept of sensitive target, Puck (1961) proposed that chromosome damage is the basic action underlying division delay. In order to explain the relationship of division delay to dose in yeast, Burns (1954) assumes that a number of independent sites in cells are specifically concerned with cell division and that division delay is proportional to the fraction of sites inactivated by radiation. Lea (1946), however, is of the opinion that target theory cannot be used to explain division delay. He further argues that lethality and delay in division are two different kinds of phenomena.

The fact that moderate doses of radiation invariably produce division delay, while at higher doses more lethal damage is caused, suggests very strongly the possibility that division delay and lethality are essentially the same phenomenon and differ only in degree. This does not imply that the damage of any one structure in the cell is responsible for both lethality and division delay. The two effects can be explained as follows.

Division delay is not a permanent effect; therefore it necessarily involves the process of recovery. Owing to the randomness of chemical-bond breakage, many types of molecules are affected. Although, at low doses of radiation, small numbers of cellular macromolecules are inactivated (Setlow and Pollard, 1962), such minor effects of radiation can be magnified through the biosynthetic process. For example, a small number of inactivated RNA molecules can in turn block the synthesis of a large number of protein molecules.

Damage to DNA, RNA, or protein molecules can also be caused indirectly by release of DNase, RNase, or proteolytic enzymes during irradiation. But so long as the cell is capable of repairing or resynthesizing these molecules, the process of recovery can take place and the cell undergoes a delayed division. Alternatively, one can also visualize a cell dividing with a damage in its genome, for this damage may not be associated with the region that is an essential coding unit for division.

At higher dose, apart from the several side effects, large deletions occur in the genome, thereby causing lethality.

As the rate of damage and repair may be different for different molecules, it is very difficult to interpret dose-delay curves unless more is known about the biochemical events underlying cell division. Our data in Fig. 18 show that initially division delay increases rapidly with the dose of radiation, and the rate of increase then falls off to a constant level. If we assume that the initial part of the curve represents the destruction of a factor essential for division, then the rate of inactivation should be proportional to the dose of irradiation. At higher doses, when all the substance is destroyed, the delay depends entirely on the rate at which this substance is resynthesized.

B. Variation of Delay with Time of Irradiation

1. Methods and Materials

Eggs were fertilized as before and allowed to develop in sea water at 15°C. Samples were taken every 2 or 10 min after fertilization and irradiated with a constant dose (1200 r). In each case the 50% cleavage time was determined in the manner described in the previous experiment.

2. Results

Figure 20 shows results of three such experiments. The following points are clear from the graph:

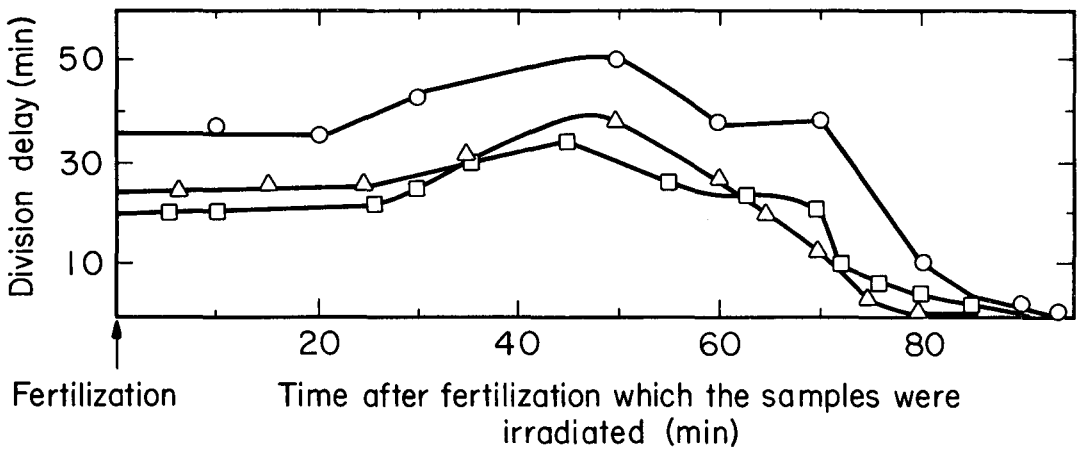
a. Delay in division is constant for some time after fertilization. Delay then begins to increase till it reaches a maximum at about 40 to 50 min after fertilization, after which it drops back to the initial value, remains constant for a while, and drops to almost zero between 70 and 80 min after fertilization. The transition from sensitive to insensitive stage is very sharp.

b. If cells are irradiated after the insensitive stage has been reached, there is no delay in the first division. Figure 21 shows the results of a similar experiment, but in this the delay was observed up to two divisions.

It is seen that if cells are irradiated after the critical point, the second division is delayed although the first division is not affected. Division delay follows the same pattern as in the first cycle.

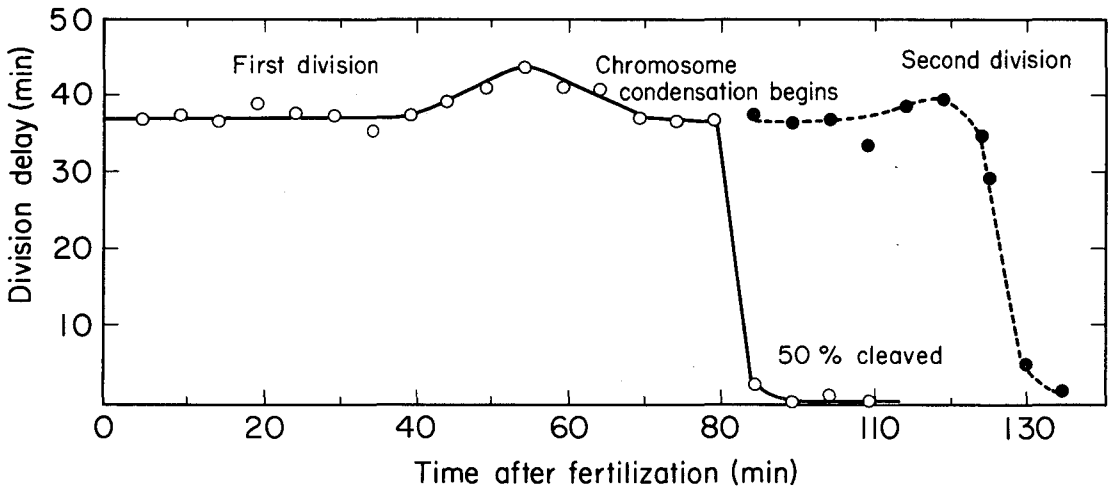
3. Discussion

As the phenomenon of reversible mitotic delay is not a permanent one, the interpretation of the experiments must take account of the process of recovery. If we agree that the initial effect of ionizing radiation is to cause some chemical changes in the cell, then as Lea (1945) points out, the recovery can be looked upon as the re-formation of the substance essential for cell division. The "enzyme release hypothesis" put forward by Bacq and Alexander



MU-32573

Fig. 20. Division delay as a function of time of irradiation; delay followed only through first division (dose 1200 r).



MU-32574

Fig. 21. Division delay as a function of time of irradiation; delay followed through second division (dose 1200 r).

(1961) is essentially the same, except that here the initial effect of the ionizing radiations is to destroy the structural organization of the enzymes, which may then destroy the substance essential for cell division. In the past few years much work has been done on the radiosensitivity of various biochemical processes. It is impossible to review all the work here; Bacq and Alexander (1961), Ord and Stocken (1961), and Kelly (1961) should be consulted. The effect of x rays on DNA synthesis has been discussed in the previous chapter, where it was shown that doses that affect cell division do not disturb the process of DNA synthesis. Recent experimental evidence is accumulating which suggests the interference of x rays with RNA synthesis. In isolated thymocyte nuclei, the incorporation of precursors for RNA is reduced, even when the irradiation doses are very low (50r) (Logan, 1959). Experiments using an x-ray microbeam have shown that irradiation of the nucleolus inhibits nucleic acid synthesis in the nucleus (Seed, 1960).

It is difficult to evaluate these results in terms of reversible mitotic delay, because most of these experiments were not designed to study this effect.

Without going into the nature of the chemical effect, Lea has used the concept of cumulative dose to explain the results found by Henshaw and Cohen (1940) on cleavage delay as a function of stage of irradiation. The dose of radiation that at any instant produces an effect quantitatively identical with the residual effect is called the cumulative dose; the residual effect is the initial effect less the recovery.

Henshaw's results are reproduced in Fig. 22. The most sensitive stage occurs 10 to 15 min after fertilization and corresponds to the period just before the onset of prophase in Arbacia eggs. The cleavage delay depends on the cumulative dose existing immediately before prophase. The further the egg is from this stage at the time of irradiation, the less the delay, because of the decay in this interval of the cumulative dose. Our results are similar to those of Henshaw, except for the periods of constant radiosensitivity just before and after the most sensitive stage. It is not possible to explain these portions of the curve on the basis of "cumulative dose." It is difficult to speculate on any other mechanism unless more is known about the biochemical processes underlying radiation injury. That irradiation of cells during mitosis, a period of depressed metabolic activity, can also give rise to a delay in second division, suggests two alternatives:

a. The release of certain hydrolytic enzymes, which may interfere with cell functions necessary for cell division. There have been reports in the literature on increased RNase activity after irradiation (Cherry et al., 1962; Roth and Eichel, 1959). In certain cases, there is not only an increase in RNase activity, but there are also changes in the relative activity of the various fractions.

b. Injury of the chromosomes in a way that depresses the formation of messenger RNA.

As pointed out earlier, the transition from sensitive to insensitive phase is very sharp. This is not very unusual and is observed in the case of many other blocking agents (Bullough, 1952). This has led to the hypothesis of an energy reservoir (Swann, 1954). The only visual process taking place

in the cell at this time is the continuation of chromosome condensation and the breakdown of the nuclear membrane. Carlson's (1942) work on grasshopper neuroblasts has also shown that irradiation after the breakdown of the nuclear membrane causes no delay. Whether the breakdown of the nuclear membrane is in any way connected with the radiosensitivity of the cell is difficult to say at this time.

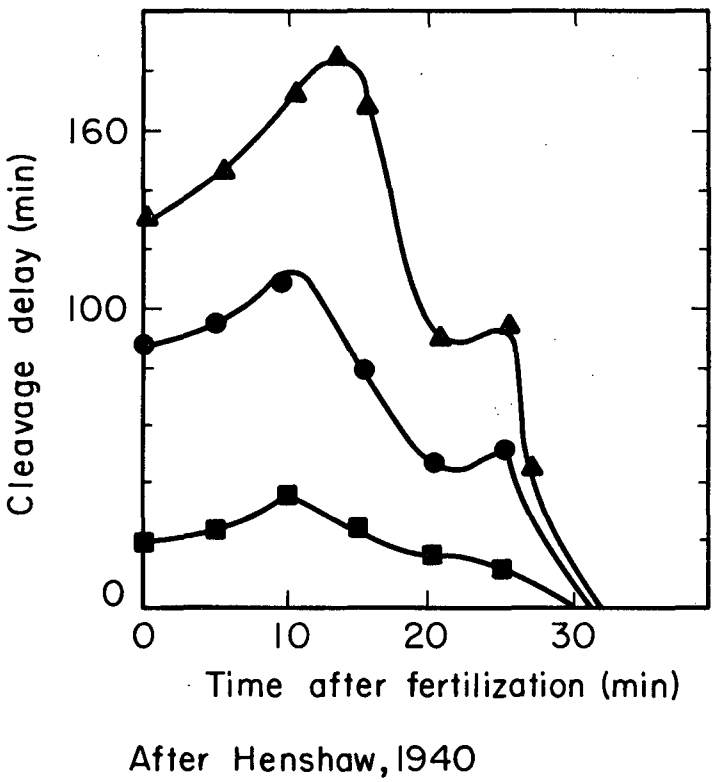


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Fig. 22. Cleavage delay as a function of time of irradiation.

V. EFFECTS OF PYRIMIDINE ANALOGUES ON THE RADIOSENSITIVITY OF MITOSIS

Radiosensitivity of a given species and strain of bacteria is known to be characteristic of that species and strain (Alper and Gillies, 1958). Various investigators have sought an explanation for this in the differences in radiation sensitivity of DNA from various sources. There seems to be some correlation between the base composition of the DNA of a particular bacterial strain and its radiation sensitivity. Thus, it was observed by Kaplan and Zavarine (1962b) that, as the guanine-cytosine content in the DNA increased, the cells became more resistant to killing by x rays. Different genetic markers of transforming DNA also show different sensitivities to inactivation by ultraviolet (Setlow and Setlow, 1962; Marmur et al., 1961). In some cases, markers with different base composition, as judged by differences in density in cesium chloride or in melting temperatures (Ganesan and Lederberg, 1963), have also shown differences in radiosensitivity (Kaplan, 1963).

Bacterial and mammalian cells, grown in the presence of certain pyrimidine or purine analogues, incorporate into their newly synthesized DNA varying amounts of these analogues (Le Page, 1960; Bieber et al., 1961; Zamenhof et al., 1956; Djordjevic and Szybalski, 1960; Berry and Andrews, 1962; Humphrey et al., 1961). Cells that have incorporated purine or pyrimidine analogues into their DNA become much more sensitive to radiation killing (Kaplan et al., 1961; Kaplan et al., 1962c; Djordjevic and Szybalski, 1960; Erickson and Szybalski, 1961; Delihas, 1962). Increase in radiation sensitivity is expressed by the ratio of the slope of the dose-log survival curves, for analogue vs natural-base-grown cells. Slope ratios of 2 have been obtained with halogenated pyrimidines(Kaplan et al., 1962c). Furthermore, Kaplan and others (1962a) have shown that E. coli grown in pyrimidine analogues has the same radiosensitivity, whether x irradiation is carried out in oxygen or in nitrogen; in contrast, radiosensitization with purine analogues completely disappears in nitrogen. These observations were confirmed in mammalian cells (Humphrey, 1963). Ragini and Szybalski (1962), working with mammalian cells, found that incorporation of thymidine analogues into these cells increases the sensitivity to ^{32}P-decay inactivation by a factor of 2. Inactivation caused by ^{32}P-decay is used as a powerful tool in the study of DNA function in phage and bacteria (Stent and Fuerst, 1960). So far, human cells with normal DNA were found to be highly resistant to this type of inactivation (Ragini and Szybalski, 1962). Study of radiosensitization following 5-bromodeoxyuridine (BUDR) incorporation has been extended to the chromosomal level by Somers and Humphrey (1963). They found a large increase of abnormal metaphases when BUDR-treated cells were exposed to x rays. The chemical mechanism by which pyrimidine analogues increase the radiation sensitivity of cells has been mainly attributed to the labilization of the phosphate ester bond proximate to the electronegative halogen atom (Djordjevic and Szbalski, 1960). Chemically, bromouracil incorporated into the DNA of bacteria is more labile than thymine (Smith, 1962a; Wacker, 1961b). In the case of uv irradiation, there is evidence that irradiated DNA containing BUDR is actually inhibitory to the photoreactivating enzyme (Rupert, 1961). This may affect the mechanism of repair from radiation injury and thus increase the radiation sensitivity.

The study of the modification of radiation sensitivity by incorporation of the various analogues into DNA is of importance in elucidating the nature of the action of x rays on biological systems.

Experiments described in this section were designed to answer the following questions:

a. Does the incorporation of analogues into sea urchin egg DNA change the radiosensitivity of these eggs, as judged by the increase or decrease of division delay?

b. Do these analogues, by themselves, cause any delay in division?

c. Is the process of chromosome condensation affected when thymidine is replaced by BUDR in the DNA?

A. Experiments with BUDR

1. Materials and Methods

Two types of experiments were done. In the first, unfertilized eggs were first irradiated (dose 1560 r); half of them were then fertilized in 0.1% BUDR soln in sea water (1 ml of packed eggs per 300 cc of BUDR soln), and the other half were placed in sea water at 15°C. The eggs were allowed to develop, and samples were fixed in Carnoy's every 5 or 10 min and scored for various phases as before. Simultaneous observations were also made on nonirradiated BUDR and normal controls. The results are shown in Fig. . 23.

In the second type of experiments, two batches of eggs were fertilized, one in 0.1% BUDR solution in sea water and the other in plain sea water. Fifty-five minutes after the time of fertilization, samples from each of these batches were irradiated for 30 sec at 52 r/sec. The irradiated eggs were allowed to develop in BUDR solution and sea water respectively. It must be pointed out here that DNA synthesis for the first division takes place somewhere between 30 and 50 min after fertilization (see also Section III). Thus, in this case, the eggs were irradiated after the DNA synthesis had taken place, whereas in the former experiment the eggs were irradiated before the period of DNA synthesis. Samples were fixed in Carnoy's, as usual, and viewed under the phase microscope.

2. Results

The results of three such experiments are summarized in Table VI.

The results in Table VI may be summarized as follows:

a. Cells grown in the presence of BUDR divide with a delay of 15 min.

b. Radiosensitivity of the cells grown in BUDR is the same as that of cells grown in normal media. This is shown by the fact that the delay in division, caused by x rays in BUDR-treated eggs, is the same as that caused by x rays in eggs placed in normal media.

c. Delay in chromosome condensation in these samples corresponds to the delay in division.

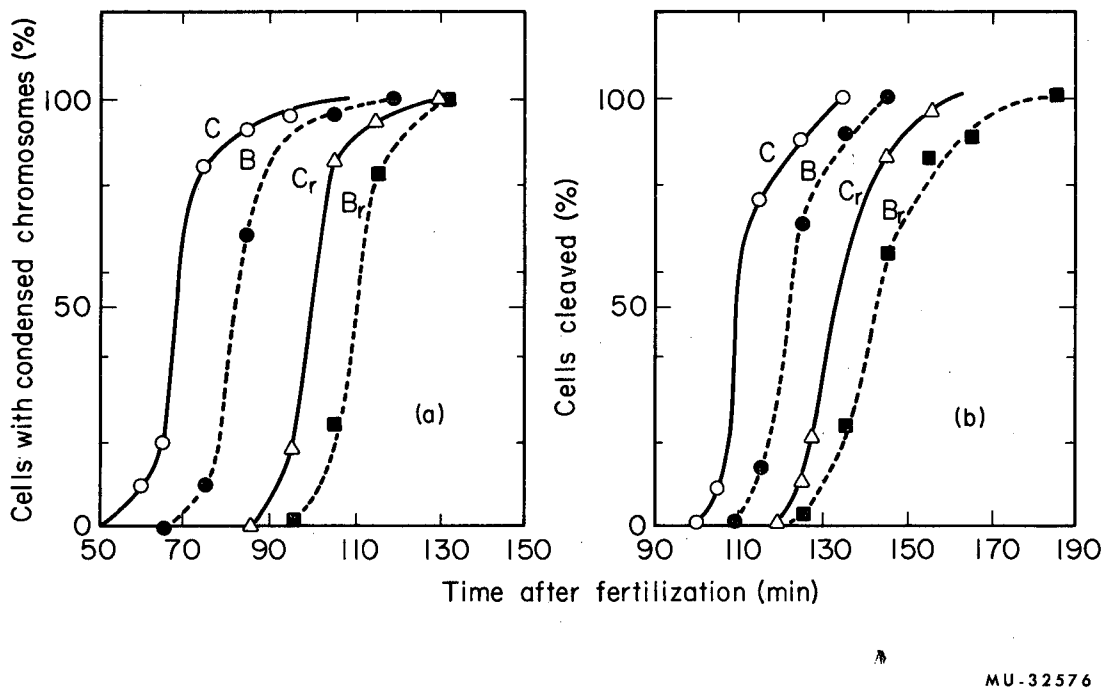

Fig. 23. (a) Chromosome condensation in different samples
(b) Cell cleavage in different samples.
C - Nonirradiated, non-BUDR samples
C_r - Irradiated, non-BUDR samples
B - Nonirradiated, BUDR samples
B_r - Irradiated, BUDR samples

Table VI. The effects of BUDR on delay in division and chromosome condensation in irradiated and non-irradiated eggs.

Experiment number	Time of irradiation	Dose	Differences between	In delay in division	In delay in chromosome condensation
1	Unfertilized eggs	1560 r	C and B	10 min	14 min
			C and C_r	23 min	31 min
			B and B_r	22 min	26 min
2	40 min after fertilization	1040 r	C and B	15 min	15 min
			C and C_r	55 min	39 min
			B and B_r	52 min	40 min
3	55 min after fertilization	1560 r	C and B	15 min	20 min
			C and C_r	24 min	30 min
			C and B_r	34 min	34 min

C - Nonirradiated, non-BUDR eggs.
C_r - Irradiated, non-BUDR eggs.
B - Nonirradiated, BUDR eggs.
B_r - Irradiated, BUDR eggs.

3. Discussion

The incorporation of BUDR into the DNA of cells increases the radiosensitivity as measured in terms of lethality, or chromosome damage (Kaplan et al., 1962a; Ragini et al., 1962; Somers and Humphrey, 1963). If however one measures radiosensitivity in terms of x-ray-induced division delay, then no such increase in this quantity is detected when BUDR is incorporated into sea urchin egg DNA. It has been observed that BUDR is incorporated into 20 to 30% of the newly synthesized DNA in these eggs (Hinegardner and Contcharoff, 1963). These results would tend to disprove the hypothesis that division delay is caused by the direct action of x rays on DNA molecules. Puck (1961) hypothesizes that reproductive death, reversible mitotic delay, and visible chromosome damage can all be explained if chromosome damage is the primary lesion produced by x rays. This would strongly indicate DNA as the main target of x rays. The fact that chromosome damage and lethality can be increased by the substitution of analogues into the DNA of the cell lends support to this hypothesis in relation to cell killing. On the other hand, in the present experiments no increase in division delay was observed in BUDR-treated cells. This would indicate that division delay is not caused by the same localized effect as the one responsible for lethal damage. It has been shown in the previous chapter that DNA synthesis also is not affected by sublethal doses of x rays; Puck (1961) disposes of these objections by pointing out that the reversible mitotic delay may be caused by damage to the mechanical process of chromosome condensation. Although our experiments show that division delay initially expresses itself in a delay in chromosome condensation, it is not possible to say whether the chromosomes or the DNA molecules are the sites of primary damage. Lea (1946) cites many examples in which there is lack of parallelism between killing and division delay. Burns (1954) and Gray (1950) show the difference in responses of lethality and division delay towards ploidy and relative efficiencies of different types of radiations. More direct evidence against the hypothesis that chromosomes are the primary sites of damage comes from our colcemide experiments, in which irradiated cytoplasm can interfere with the process of condensation in the nonirradiated nucleus.

It is interesting to note that incorporation of BUDR into the DNA of sea urchin eggs causes a delay in division in these eggs. Several possible mechanisms could be suggested to explain this delay:

a. Change in the rate of DNA synthesis

It is quite conceivable that BUDR is not incorporated into DNA with the same efficiency as thymidine. The cell may not contain the necessary enzymatic mechanism for phosphorylating bromodeoxyuridine to the triphosphate form. These enzymes may be induced after a certain time. Although several workers have reported the incorporation of BUDR into bacterial, virus, and mammalian DNA, there is no mention of the fact that this causes any decrease in the rate of synthesis of DNA. Bessman et al. (1958), working with a cell-free system, have shown that in *E. coli* many natural bases can be substituted by their analogues so long as there is no interference in hydrogen bonding. Also, it is known that 5-bromodeoxyuridylate is readily converted into the triphosphate form by *E. coli* preparations (Zamenhof and Griboff, 1954). If *E. coli* results can be extended to sea urchin eggs, then it seems most unlikely that the substitution of BUDR into the DNA causes any change in the time or rate of DNA synthesis.

b. Changes in the base composition

Substitution of BUDR into DNA of cells could also lead to division delay, if we assume that change of sequence of bases in DNA causes the production of defective messenger RNA.

Shapiro and Chargaff (1960), while studying acid hydrolysis of BUDR-substituted DNA in E. coli, showed that there was a drastic change in the sequence of the bases. On the other hand, when "nearest neighbor" nucleotide analyses of the bromouracil-substituted and normal E. coli were compared, the results were indistinguishable (Trautner, Swartz, and Kornberg, 1962). It has also been shown that the transforming activity of BUDR containing DNA is the same as that of normal DNA. Thus we see that the evidence regarding the genetic change brought about by BUDR substitution is contradictory.

Kaplan et al. (1961), in their work on radiosensitization owing to BUDR incorporation, do not indicate the effect of BUDR on cell growth. Zamenhof and co-workers (1956), while studying E. coli containing unnatural pyrimidine in DNA, reported that a BUDR concentration of 100 μg/ml, cells form abnormal colonies. This change is not permanent; upon transfer to normal media, normal colonies appear.

c. Interference with the process of chromosome condensation

It is known that BUDR in sufficiently high concentrations can produce chromosome damage (Hsu and Somers, 1961, 1962). The presence of bromouracil in the DNA has also been found to change the hydrogen bonding as judged by a slight increase in the melting temperature of the substituted DNA (Kit and Hsu, 1961). Our results show a delay in chromosome condensation in the cells treated with BUDR. It is thus quite conceivable that the substitution of BUDR in place of thymidine interferes with the process of chromosome condensation. If Anderson's view (1956) of chromosome condensation were accepted, this interference would not be expected. According to Anderson's hypothesis, chromosome condensation is caused by the interaction of polycationic compounds with the phosphate groups of the DNA. His hypothesis finds support in observations reported by many workers. Philpot and Stanier (1957) could induce prophase-like images in isolated nuclei by increasing the ionic strength of the surrounding media, by addition of Mg^{++} ions, or by protamine or histones. Polyamines such as agmeline, spermine, and spermidine have been found to cause condensation in nuclei isolated from rat liver cells (Anderson, 1960). These studies were extended to living L mouse cells by Whitfield (1962). Polyamines also cause chromatin condensation in lateral roots of Vicia faba (Anderson, 1960).

Substitution of BUDR in place of thymidine should not alter the availability of phosphate groups, and thus no interference of chromosome condensation should be detected. Our results, therefore, do not support Anderson's hypothesis of chromosome condensation.

Although the mechanism of chromosome condensation is not clear, this still does not rule out the possibility that the delay in division is a result of interference with chromosome condensation.

B. 5-Fluorodeoxyuridine Experiments

There are many reports in the literature on the cytological and chemical effects of FUDR on cells (Taylor, 1962; Berger and Witkus, 1962; Bell, 1962). It is known that FUDR is an inhibitor of the enzyme thymidylate synthetase (Cohen et al., 1962). The conversion of deoxyuridylate to thymidylate is thus blocked, thereby inhibiting DNA synthesis. This block can be overcome by supplying an excess of thymidine. FUDR, after breakdown to FU, is also incorporated into RNA (Cordon and Stashelin, 1958). This incorporation can be prevented by giving the cells an excess of uridine.

Experiments with FUDR were done to determine the effects on cell division of FUDR incorporation into sea urchin egg RNA. Excess thymidine was supplied to the cells to avoid any disturbance in DNA synthesis.

1. Experimental Procedure

Three batches of eggs were fertilized. The first contained 10^6 eggs in 200 cc of sea water, the second contained 10^6 eggs in 100 cc of 0.02% thymidine plus 100 cc of 0.001% FUDR, and the third sample contained 10^6 eggs in 200 cc of FUDR solution. The eggs were allowed to develop at 15°C as far as the gastrulation stage. Times for first, second, and third divisions were determined for each sample.

2. Results

a. In three such experiments no delay was observed in the first, second, or third division with the FUDR or FUDR plus thymidine.

b. The development of the embryos in eggs treated with FUDR plus thymidine was normal.

c. Eggs grown in solution containing only FUDR did not develop beyond the sixteen-cell stage.

3. Discussion

Our results are in agreement with those of Nemer (1962), who, working with sea urchin *Paracentrotus lividus*, found that FUDR stopped the development of the embryos at the eight-blastomere stage. He further showed that, if thymidine was added to the solution containing embryos in FUDR, the embryos developed as far as the gastrulation stage.

A block in the development after the sixteen-blastomere stage of embryos exposed to FUDR in the absence of thymidine can be explained on the basis that there is enough thymidine in these eggs to support DNA synthesis as far as the sixteen-blastomere stage.

The result--that the eggs in FUDR plus thymidine media can develop as far as the gastrulation stage--is a little surprising. Gross and Gilles (1963), in their study on the effects of actinomycin D on the early development of sea urchin eggs, have shown the existence of RNA synthesis in the early stages of development. Nemer (1962) indicated that the embryo is capable of incorporating uridine, but this incorporation is markedly inhibited by the presence of FUDR. In the light of these findings our results would indicate that, although the presence of FUDR affects RNA synthesis, this does not in any way interfere with cell division.

Nemer et al. (1963) and Wilt and Hultin (1962), have shown the difference in phenylalanine incorporation by polyuridylic acid in ribosomes from fertilized and unfertilized eggs. They propose that functional messenger RNA is present only after fertilization. It is also known that protein synthesis in these eggs starts right after fertilization (Gross and Gilles, 1963; Nemer, 1963) and that, although actinomycin D inhibits the uptake of uracil, there is no appreciable effect on protein synthesis (Gross and Gilles, 1963). Cell division in the early stages also proceeds quite normally. This is quite unexpected in view of the antimitotic action of actinomycin D on tissue-culture cells (Reich et al., 1962).

Thus, it seems that although RNA synthesis does take place during development, it is perhaps not a necessary requirement for the early cell divisions in these eggs. All the information needed to synthesize protein is already present in the egg at fertilization. This is not surprising if one considers that sea urchin eggs continue to divide for several hours even after the nucleus has been removed (Harvey, 1956).

The role of the newly synthesized RNA is not clear. A suggestion has been made by Gross and Gilles (1963) that this RNA is the messenger RNA required for differentiation.

VI. SUMMARY

x-Irradiation of egg, sperm, or zygote of sea urchin Strongylocentrotus purpuratus causes a delay in the visual onset of chromosome condensation. This delay corresponds exactly to the delay in division in these eggs.

Detailed cytological investigation of the irradiated eggs shows that the processes of pronuclear fusion and of aster and spindle formation are not affected by x rays. Duration of prophase, metaphase, anaphase, and telophase are the same in the irradiated and nonirradiated samples.

Chromosome condensation in the early prophase stage can be temporarily blocked by irradiating the eggs during that stage.

Mercaptoethanol was used as an experimental tool to obtain two types of cell populations. In both types division was temporarily arrested; but in one the process of chromosome condensation had gone to completion whereas in the other it had not taken place. The cells in which the chromosomes had already condensed were not affected by x irradiation, whereas irradiation of the cells with uncondensed chromosomes caused a delay in cell division. This, together with the above observations, points strongly to the role of chromosome condensation in radiation injury. Although the site of primary lesion is not known, division delay due to x irradiation is expressed as a delay in chromosome condensation.

Fusion of the sperm and the egg nuclei was prevented by placing the eggs in 0.01% colcemide soln. It was thus possible to study the effect of x rays on the condensation of the chromosomes in the egg or sperm nuclei. Irradiation of the sperm delayed the condensation of the chromosomes in the sperm nuclei but had no effect on the condensation of the egg chromosomes.

On the other hand, irradiation of the unfertilized egg delayed the process of chromosome condensation in both the egg and sperm nuclei. From these experiments, it is inferred that both the nucleus and the cytoplasm are radiosensitive.

Division delay was studied as a function of dose of x rays and time of irradiation. It was shown that the most sensitive stage is just before the onset of the visible prophase.

Sublethal doses of x rays have no effect on the process of DNA synthesis. On the other hand, the experiments indicate that the mitotic activity may in some way regulate DNA synthesis.

Irradiation of the eggs exposed to 5-bromodeoxyuridine did not show any enhancement of division delay over those grown under normal conditions. These results contradict the hypothesis that division delay is caused by the direct action of x rays on DNA molecules.

FUDR has no effect on the first three cell divisions in sea urchin eggs.

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APPENDIX I

Symbols and Abbrevations Used in the Text

I — Interphase:
Nuclear membrane intact. Chromosomes not visible.

EP — Early prophase:
Nuclear membrane intact. Separation of the centriols. Chromosomes beginning to be visible.

LP — Late prophase:
Nuclear membrane intact. Chromosomes well defined.

PM — Prometaphase:
No nuclear membrane. Chromosome arrangement random.

M — Metaphase:
No nuclear membrane. Chromosomes lined up at the equitorial plate.

A — Anaphase:
Daughter chromosomes separate to move toward the pole.

T — Telophase:
Daughter chromosomes are at the poles. Chromosomes have begun to uncoil and the nuclear membrane has begun to form. Cleavage furrow begins to form.

C — Cleavage:
Interphase nuclei in the daughter cells clearly visible.

APPENDIX II

Method of Determination of Duration of Various Phases of Cell Division

From the data of Table I it is possible to plot the percentage of cells in and past a particular stage of cell division. This is done in Fig. 24 for irradiated and nonirradiated samples. Curve EP represents the percentage of cells in and past early prophase; similarly other curves represent cells in or past that stage. If the 50% point is chosen to calculate the duration of different phases, then the average time spent in early prophase is the difference between the two points (on the time axis) where the 50% line cuts the curves EP and LP (83-79 = 4 min). Similarly, duration of other phases can be calculated.

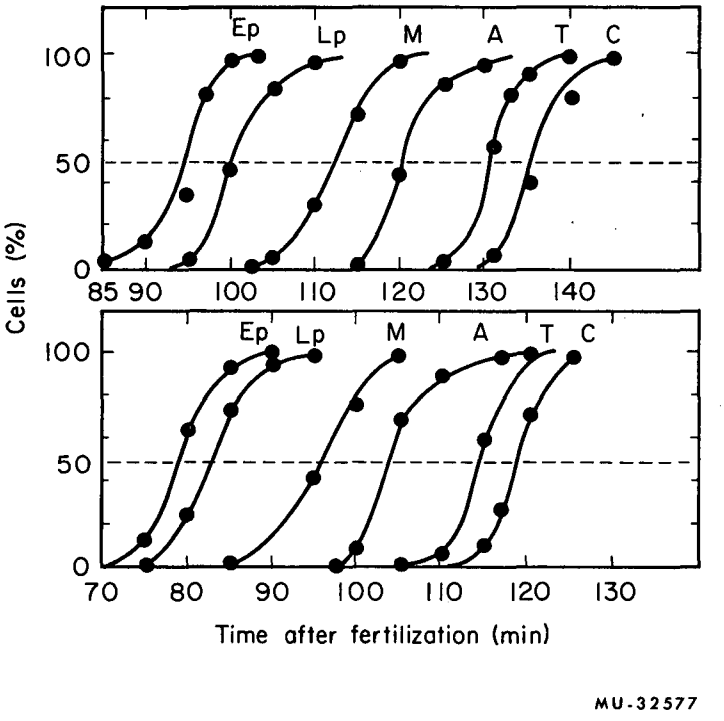

Fig. 24. Top, irradiated eggs. Bottom, control (nonirradiated) eggs. For explanation of symbols, see Appendix I.

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