

NUCLEAR-CYTOPLASMIC RELATIONS IN THE MITOSIS OF SEA URCHIN EGGS

III. γ -Ray-Induced Damage to Whole Eggs and Nucleate and Anucleate Half-Eggs

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ABSTRACT

Sea urchin eggs were cut into halves. The nucleate and anucleate halves and whole eggs were irradiated with γ -rays and then fertilized with normal sperm. The first mitosis of the diploid half-egg was more delayed than the division of the whole egg. There was a small, but highly significant, delay of the mitosis of the haploid half-egg, thus demonstrating cytoplasmic sensitivity to ionizing radiation. Since the sensitivity of nucleate cells is influenced by cytoplasmic volume, the problem of the role of cytoplasm in repair is considered in relation to these data and other reports in the literature.

INTRODUCTION

A variety of evidence favors the view that the structures most sensitive to radiation are usually located either in or near the nucleus. Nonetheless, cytoplasmic radiation damage has been demonstrated in a number of types of cells (reviewed by Zirkle, 1957; Smith, 1964; Lessler, 1964; see also Brown and Zirkle, 1967; Burchill and Rustad, 1969).

A classic study on sea urchin eggs by Henshaw (1938) was consistent with the view that the cytoplasm was not sensitive to ionizing radiation. Henshaw prepared anucleate half-eggs from *Arbacia* by centrifuging the cells into halves. When the X-irradiated anucleate halves were fertilized with normal sperm, no mitotic delay could be detected. This experiment was confirmed by Blum et al. (1951), and a preliminary study in our

laboratory failed to detect any cytoplasmic effect even at much larger doses than those employed by the earlier workers (Rustad et al., 1963).

The centrifugation procedure used to obtain anucleate sea urchin eggs is known to stratify cytoplasmic organelles according to their densities (see Harvey, 1956). Hence, the composition of the anucleate half is quite different from that of either the nucleate half or the whole cell. One apparent consequence of this abnormal distribution of cytoplasmic components is a considerable delay in the division of the anucleate half after it is fertilized (see Harvey, 1956). In Henshaw's original study, the unirradiated haploid halves divided later than the irradiated whole eggs (Henshaw, 1938). Therefore, if the factors involved in radiation-induced mitotic delay were independent of those causing

the division delay of the centrifuged haploid cells, radiation damage could not have been detected. In addition, a purely hypothetical consequence of the centrifugation procedure might be the flotation into the nucleate half of organelles which were capable of releasing a "radiotoxin" when irradiated.

Since the studies on centrifuged sea urchin eggs did not agree with some results on other cells, we have investigated this problem by another method. Sea urchin eggs can be cut into approximately equal halves with fine glass needles. Following fertilization, the diploid half-egg divides at the same time as a whole egg, while the haploid half is slightly delayed (Rustad et al., 1970). A pilot study with small numbers of anucleate eggs had indicated that the cytoplasm was sensitive to X-ray-induced damage leading to mitotic delay (Rustad, 1961). With improved experimental techniques and the availability of three persons trained in the cutting procedure, it was possible to investigate the relationship between the dose of γ -rays administered to whole eggs and nucleate and anucleate half-eggs, and the mitotic delay. Furthermore, it was possible to study the mitosis of a limited number of partial eggs in which the cytoplasmic composition had been varied by stratifying eggs by centrifugation and cutting along visible bands of organelles.

MATERIALS AND METHODS

Gametes were obtained from the sea urchin *Arbacia punctulata* by stimulating the animals with a 12 v alternating current (see Harvey, 1956). The microsurgical procedures have been described in detail elsewhere (Rustad et al., 1970). Since the eggs for each experiment were cut freehand by either two or three different persons using different microscope lamps, glass microneedles, and agar-coated Syracuse dishes, certain procedures were used to randomize any possible systematic difference among different groups of cells. Each person separated whole eggs and nucleate and anucleate half-eggs into different groups and stirred each group in order to mix eggs which had been cut early and late and which had been obtained from different parts of the dish. Then the individual groups were subdivided into three equal subgroups and transferred into separate dishes with a braking pipette. Each person contributed an equal fraction of the different kinds of egg which he had handled to each control and experimental dish. Thus, the experimental array consisted of three sets of three dishes, providing one sample of each type of cell for a control and for two samples to be irradiated at two different doses. In different experiments individual

dishes contained between 25 and 40 cells, usually 30-35. Irradiations were performed with a ^{137}Cs source which provided 663 kev γ -rays at a dose rate of 5000 R/min in 1964 and 4900 R/min in 1965.

In some experiments the cellular organelles of the eggs were stratified by centrifuging for 10 min at 10,000 g at the interface between seawater and 1.1 M sucrose (see Harvey, 1956). The eggs were quickly removed from the centrifuge tubes and mixed with seawater in the cutting dishes. Anucleate fragments either containing or lacking the "mitochondrial layer" were prepared by cutting the eggs before the stratified organelles became redistributed by brownian motion (see Fig. 1 in Rustad et al., 1970).

RESULTS

Effects of Prefertilization Irradiation

Whole eggs and nucleate and anucleate half-eggs were fertilized. Unirradiated whole eggs and diploid half-eggs divided at the same time, while haploid half-eggs always divided some 5-15 min later (e.g. Fig. 1).

Following γ -irradiation and subsequent fertilization with normal sperm, all three types of cells exhibited mitotic delay. The diploid half was delayed more than the whole cell, and both kinds of diploid cells were substantially more delayed than the haploid half which had been irradiated while no nucleus was present (Fig. 1). The times when 50% of the fertilized eggs had divided and the average division times of those cells which were able to divide were determined from the cumulative distribution curves (e.g. Fig. 1). When these parameters of the division times were plotted against dose, curves such as those shown in Fig. 2 were obtained. This figure illustrates that the nucleate halves are considerably more sensitive than the whole eggs and reveals the small (but highly significant) sensitivity of the anucleate half-eggs.

These experiments with γ -rays have demonstrated (a) an effect of cytoplasmic volume on the sensitivity of nucleated cells, and (b) cytoplasmic sensitivity.

Radiation Sensitivity of Stratified

Anucleate Eggs

The cytoplasmic contents of the eggs were stratified by centrifuging the cells at 10,000 g at an interface between seawater and 1.1 M sucrose. This treatment produced sharp stratification of the cytoplasmic organelles without pulling the

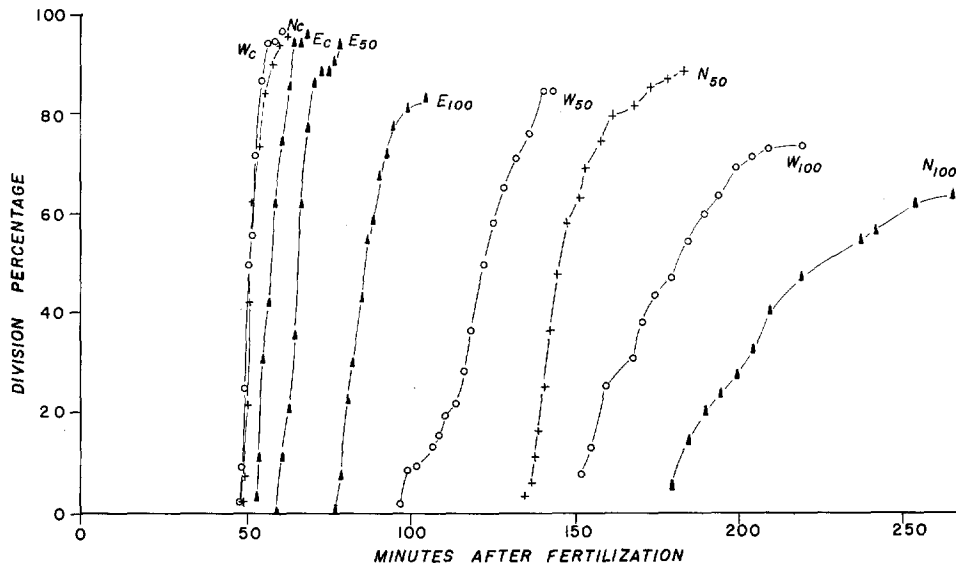


FIGURE 1 Cumulative distributions of division times of whole eggs (*W*) and of nucleate (*N*) and enucleated (*E*) half-eggs following fertilization with normal sperm. The subscripts *C*, 50, and 100 denote unirradiated eggs and eggs which were irradiated with 50 or 100 kR of γ -radiation, respectively. Thus, the irradiated diploid eggs (W_{50} , W_{100} , N_{50} , and N_{100}) contained an irradiated female pronucleus, while the irradiated haploid eggs (E_{50} and E_{100}) were purely cytoplasmic half-cells when irradiated.

eggs into halves. Two types of anucleate fragments were prepared by cutting adjacent to visible bands of organelles. The first was an anucleate half-egg which did not contain the mitochondrial layer. It was equivalent to the anucleate halves which can be prepared by pulling the cells into halves with further centrifugation (see Harvey, 1956). The second type of anucleate fragment was larger and contained the mitochondrial layer and a very small amount of the clear layer.

After fertilization, the haploid halves lacking the mitochondrial layer divided 40–70 min later than the whole eggs. In contrast, the larger haploid fragments containing the mitochondrial layer were delayed only 5–10 min (see Rustad et al., 1970). Thus, the delay of the fragments containing the mitochondrial layer is comparable to the delay found in uncentrifuged anucleate eggs after fertilization.

100 kR of γ -radiation did not induce detectable mitotic delay in the halves which lacked the mitochondrial layer (except in one experiment in which the unirradiated halves divided exceptionally early). In contrast, the fragments containing the mitochondrial layer were sensitive to these doses of γ -rays, and this sensitivity was comparable

to the sensitivity of uncentrifuged enucleated half-eggs.

It was impossible to determine whether or not the halves lacking the mitochondrial layer had been damaged, because unirradiated cells of this composition divided later than other types of anucleate cells which had been γ -irradiated. These microsurgical experiments on centrifuged eggs have demonstrated cytoplasmic radiation sensitivity and also indicate that a cytoplasmic effect was not detected in Henshaw's (1938) experiments, because the mitochondrial layer was absent from his anucleate eggs.

DISCUSSION

Normally, the division times of whole and half-sized diploid eggs are identical, while the haploid half-sized eggs are delayed in their first division; however, ploidy does not influence the mitotic rate during subsequent divisions (Rustad et al., 1970). In the present study both the cytoplasmic volume and the presence or absence of a nucleus influenced the radiation sensitivity. Prefertilization irradiation of the nucleate half-egg resulted in a larger mitotic delay than occurred when the same dose of γ -rays was administered to the

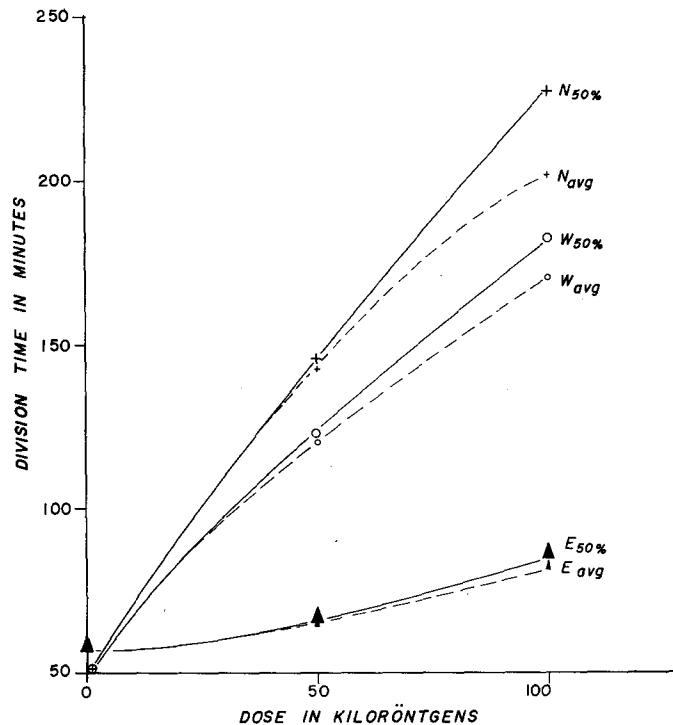


FIGURE 2 The relationship of division time to the dose of γ -radiation administered to whole eggs (*W*) and to nucleate (*N*) and enucleated (*E*) half-eggs following fertilization with normal sperm. The times when 50% of the eggs in each of the population shown in Fig. 1 had divided are shown. In addition, the average division time (subscript avg) of those cells in each population which were able to divide are plotted, because many cells did not divide after large doses.

whole egg. Hence, reduction of the size of a nucleate cell increases the radiation sensitivity. The irradiated anucleate half-egg exhibited much less mitotic delay after fertilization than the whole egg. Hence, the cytoplasm is sensitive to damage by ionizing radiation, but damage to some nuclear or perinuclear structure appears to have a much larger influence on the time of mitosis.

The Influence of Cytoplasmic Volume on the Sensitivity of Nucleate Cells

The extreme sensitivity of the nucleate half-egg in comparison with the whole egg does not seem explicable in terms of removing a quantity of some cytoplasmic factor normally required for mitosis, because quarter-sized cells and even some cells as small as $\frac{1}{80}$ of the normal volume can divide on schedule (Rustad and Rustad, 1960; Rustad et al., 1970). It seems more likely that the cytoplasmic volume or surface area influences the rate of recovery from radiation damage. Both

unfertilized eggs (Henshaw, 1932) and fertilized eggs (Failla, 1962) exhibit recovery from the damage by X-rays which leads to mitotic delay.

Normally, little protein synthesis occurs in the egg until shortly after fertilization (reviewed by Tyler 1966; Gross, 1967), but γ -irradiation stimulates the incorporation of amino acids into proteins in unfertilized eggs (Rustad, 1967). The messenger RNA for protein synthesis is already present in the unfertilized egg, and the inhibition of nuclear RNA synthesis with actinomycin D has no noticeable effect on either the rate of post-fertilization protein synthesis (Gross and Cousineau, 1964) or the magnitude of the radiation-induced mitotic delay (Rustad and Burchill, 1966). Puromycin (an inhibitor of protein synthesis at the ribosome level) appears to inhibit the recovery of irradiated eggs both before fertilization (Rustad et al., 1966) and during the first division cycle (Rustad and Burchill, 1966; Failla and Rustad, 1970). Therefore, it seems reasonable

to speculate that the difference in sensitivity between the whole egg and the nucleate half-egg may be due to the difference in the rate of a repair process which is dependent on the total amount (rather than the concentration) of proteins synthesized by cells of different volumes.

As yet, there has been no direct test of the possibility that the influence of cell volume is primarily on the capacity to produce "recovery proteins"; however, further experiments are in progress (e.g. Rustad and Greenberg, 1970). The recovery from damage leading to regeneration delay in the ciliate protozoan *Stentor* (Burchill, 1968) and to mitotic delay both in mammalian cells in tissue culture (e.g. Walters and Petersen, 1968; Doida and Okada, 1969) and in plant meristematic cells (Van't Hof and Kovacs, 1970) also may require protein synthesis.

Cytoplasmic Sensitivity

Cells containing nuclei at the time of irradiation were much more sensitive than those lacking nuclei (Fig. 2). However, the anucleate eggs did show significant sensitivity to 50 and 100 kR of γ -radiation, as measured by the mitotic delay after insemination with normal sperm. The effects of larger doses could not be studied because the eggs became difficult to fertilize, and sometimes stuck to the glass dish and/or lysed. The shape of the sensitivity curves for anucleate cells seemed qualitatively different from that of the sensitivity curves for nucleate ones: the inflection of the dose-delay curve was consistently positive rather than negative (Fig. 2). However, the mitotic delays resulting from cytoplasmic irradiation were too small to permit an exact determination of the shape of the dose-response curves.

There is no evidence that sea urchin eggs might repair the cytoplasmic damage that leads to mitotic delay. Considerable damage may occur, but the small cytoplasmic response observed could be the result of either a very efficient repair system or an unusually long period of time available for recovery before some critical event very early in the mitotic cycle of the haploid cells (which also have a longer division time than the diploid ones). Identical damage to irradiated sperm is also expressed as less mitotic delay in haploid than in diploid division cycles (see Rustad et al., 1967), and the question of recovery during the mitotic cycle of cells of different ploidies and

volumes will be discussed in detail in a subsequent publication.

Sensitivity of Anucleate Cells with Different Cytoplasmic Compositions

The mitochondrial layer of centrifuged eggs contains other structures, such as vitally stainable granules, which may be lysosomes (Kojima, 1959), and some mitochondria are found throughout the centrifuged cell (e.g. Geuskens, 1965; Anderson, 1970). Anucleate fragments containing the mitochondrial layer divided at approximately the same time after fertilization as uncentrifuged anucleate eggs, and mitotic delay resulted from cytoplasmic radiation damage. In contrast, the postfertilization division time of the anucleate lacking the mitochondrial layer was of the order of twice the duration of the first mitotic cycle of uncentrifuged halves, and no radiation-induced mitotic delay could be demonstrated.

However, measurements on anucleate eggs demonstrated that neither irradiated nor unirradiated cells lacking the mitochondrial layer divided until after the cleavage of the uncentrifuged irradiated cells or of irradiated fragments containing the mitochondrial layer. Therefore, if those factors which delay the division of the eggs lacking the mitochondrial layer were independent of the factors which cause radiation-induced mitotic delay, the radiation effect would be undetectable.

In view of the present observations, it can be concluded that previous studies on eggs centrifuged into halves (Henshaw, 1938; Blum et al., 1951; Rustad et al., 1963) failed to detect the cytoplasmic sensitivity to ionizing radiation because the anucleate cells lacked the mitochondrial layer.

The Generality of Cytoplasmic Susceptibility to Ionizing Radiation

In contrast to the present results with γ -radiation, the cytoplasm of the sea urchin egg was found to be extremely sensitive to UV radiation (Rustad et al., 1965). Since the details of the UV sensitivity will be treated in a manuscript presently in preparation, the present discussion will be confined to selected comparisons with other studies concerning ionizing radiations.

A classical form of partial cell irradiation ex-

periment involves shielding the nucleus from short-range α -particles (sometimes just by positioning a large cell with an eccentrically located nucleus). The cytoplasmic effects of α -particles on mitosis are small or undetectable in fern spores (Zirkle, 1932), sea urchin eggs (Miwa et al., 1939), and chick heart fibroblasts (Munro, 1959). Cytoplasmic effects on division were detected in the alga *Zygnema* by Petrova (1942) at doses of α -particles some 700 times greater than those required when the nucleus was also irradiated. The susceptibility of insect eggs to the lethal effects of X-rays (Ulrich, 1955) or α -particles (von Borstel and Rogers, 1958) is much higher when the nucleus rather than the cytoplasm is irradiated.

In the studies cited in the preceding paragraph only *part* of the cytoplasm was irradiated, so the results could be influenced by the unirradiated cytoplasm in each cell. Cytoplasmic damage is more obvious in some studies in which *all* of the cytoplasm was irradiated (e.g. nuclear transplantation experiments in amoebae indicated that the nucleus is only 2.4 times "more sensitive" to X-radiation than is the cytoplasm [Ord and Danielli, 1956]). Anucleate fragments of the alga *Acetabularia* are more easily killed with X-rays than the nucleate ones (e.g. Bacq et al., 1957). Therefore, radiation may damage components of the cytoplasm which can normally be replaced by a nucleus (even by a nucleus damaged by radiation). In some strains of giant multinucleate amoebae the fusion of unirradiated cytoplasm to irradiated cells leads to the recovery from a supra-lethal dose (e.g. Daniels, 1955).

In the present experiments the lower sensitivity of the whole egg in comparison with the nucleate half-egg was interpreted as the ability of the larger volume of irradiated cytoplasm in the whole egg to permit a more rapid rate of repair of the radiation damage. Comparisons between nucleate and anucleate half-eggs suggest that, if one took the ratios of the mitotic delays induced by 100 kR as a minimum value, the sensitivity of the *Arbacia* egg cytoplasm would be 15% of that of the nucleus.

A comparison among the data on *Acetabularia*, amoebae, and sea urchin eggs would suggest that both the nucleus and the cytoplasm can be damaged by ionizing radiation and that nuclear factors may contribute toward the recovery of the cytoplasm and vice versa.

Special thanks are due to Dr. O. F. Nygaard for his many helpful suggestions concerning the preparation of the manuscript.

These experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts.

This research was supported by contract W-31-109-ENG-78, Report Number C00-78-211, with the Division of Biology and Medicine, United States Atomic Energy Commission, and contract Nonr 4785(00) between the Office of Naval Research and the Marine Biological Laboratory, Woods Hole, Massachusetts.

Received for publication 12 February 1970, and in revised form 25 January 1971.

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