THE EFFECT OF β IRRADIATION ON THE RESPIRATION AND MORPHOLOGY OF *MELANOPLUS DIFFERENTIALIS* EMBRYOS

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Since the days of Flemming, it has been known that the chromatin component of the cell nucleus stains differentially during the various stages of mitosis. Foley (1) used a modification of Flemming's triple stain and was able to promote consistent results in staining chromosomes. This combination of safranine, gentian violet, and orange gold stains the chromatin of the resting nucleus blue; the spireme and chromosomes in prophase, blue; the chromosomes in metaphase, brilliant red; and those in anaphase and telophase, purple.

The very fact that chromatin material stains differentially in these various stages suggests that we are not dealing with nucleic acids alone. If we are dealing with nucleic acids, the structural composition or the chemical affinity to the dyes changes during the process of cell division.

In the literature (2-9) every stage of mitosis, as well as the resting cell itself, has been labeled the most susceptible to ionizing radiations. This indicates that we cannot generalize the effects of irradiation on a given cell. Yet every cell, whether plant or animal, contains ribonucleic acid and desoxyribosenucleic acid.

Considering these facts, it is hard to believe that effects produced by ionizing radiations are dependent only upon the nucleo-proteins. If this were true, the injury sustained by a cell would always be demonstrable at the same stage of mitotic activity.

In a previous communication (10) we have shown that morphologically indistinguishable cells react differently to a given dosage of radiation. Sparrow (11) has shown that there is a close correlation between the increment of desoxyribosenucleic acid content of spore chromosomes and the susceptibility to X irradiation. In a second communication (12) we have shown that pyknotic nuclei contain a large amount of desoxyribosenucleic acid. The mitotic stages, as well as the resting cell, also contain the same acid but in larger amounts as the mitosis progresses. These results were found through the use of Feulgen's reaction. However, since Feulgen's reaction is limited in specificity to desoxyribosenucleic acid content, its use is inappropriate in determining any additional effects brought about by the irradiation of chromosomes.

The present work was undertaken to determine the physical or chemical changes in irradiated nuclei. Foley's modification of Flemming's triple stain was chosen because of its usefulness in differentiating between the various stages of mitosis. This stain, unlike hematoxylin, is translucent, thereby permitting one

to study the internal structure of the chromosomes and observe effects other than those on desoxyribosenucleic acid.

Material and Methods

Melanoplus differentialis eggs of known chronological and morphological age were used (13). Care in handling the material was strictly adhered to, as reported in a previous communication (10). The chronological and morphological age of the embryos was 10—16 days (prediapause). They were kept at 25° ± 0.1° C.

The sources of β radiation were P^{32} and Sr^{89}. The embryos were treated with carrier-free P^{32} containing 2.33 μc per 0.1 cc. calculated to give 3.1 r per hour, and exposed to the above source from 2½ to 24 hours. Those used for respiration studies were exposed to P^{32} and Sr^{89} solutions containing from 2.25 μc to 2930 μc for periods of 1 to 6 hours. Equivalent amounts of phosphate or strontium chloride were placed in the vessels containing the controls.

In the respiration studies the eggs were irradiated in the Warburg vessels while respiration was being measured, or they were irradiated first, and then freed from irradiating material before measurement of respiration. The irradiation was stopped by washing the eggs with 0.1 N HCl and then with distilled water, followed by dechorionation of the outer egg shell with 2.5 percent NaOCl (14). After the removal of the chorion the eggs were washed with distilled water, with 10 percent citric acid solution, and again with distilled water. Counts made with the aid of a methane Nucleometer indicated no radioactivity present on the exterior or interior of the eggs after such treatment. This is evidence that the effects observed could be attributed exclusively to ionizing radiations and not to phosphorus or strontium.

The embryos used for cytological studies were dissected out of the eggs in Belar solution and fixed individually, immediately after removal from the egg, in Allen's B-15 at 37° C. Dehydration of the embryos was done with the usual alcohol series. The embryos were cleared in aniline oil and embedded and sectioned in paraffin. The sections were cut at 7 μ and mounted serially on the slides. These were then hydrated, treated with 0.5 percent osmic acid for 1 hour, and stained with Flemming's triple stain as modified by Foley (1). They were cleared in carbolxylol and mounted in clarite. When necessary, stained slides were hydrated and placed in 50 cc of water containing 3 drops of 3 percent H_2O_2 for 10 minutes. This procedure completely bleached the osmophilic material. A very small amount of the triple stain was affected by this method.

Cytological studies were made with the aid of an E. Leitz Ortholux microscope with an apochromatic oil immersion lens of 1.32 N.A.

A 35-mm micro-Ipso attachment with appropriate filters was used to obtain the photographs recorded in figures 1 through 9.

Results

The grasshopper egg contains a yellow and a white cuticle which together form an impervious barrier to many substances that are physiologically toxic. For example, the em-

TABLE 1.—Q_{O_2} PER EGG PER HOUR WHILE BEING EXPOSED TO β RADIATION FROM A P^{32} SOURCE

Age of eggs (days of prediapause)	μc P^{32} (per vessel)	Av. Q^{O_2} controls (cu mm/eggs/hr)	Av. Q^{O_2} irradiated eggs (cu mm/eggs/hr)	Duration of experiment (hrs)	Total r equiv. (during expt. time)	No. of exp.	Irradiated first then Q^{O_2} measured
10	78.6	.349	.389	6	642.0	3	----
14	12.4	.322	.334	2	34.2	3	----
14	21.6	.385	.382	2	59.0	4	----
14	173.0	.353	.372	5	1215.0	3	----
14	102.0	.325	.326	2½	347.5	3	----
15	2.25	.302	.370	3	9.2	6	----
12	10.6	.346	.358	2	29.2	3	.298
13	10.2	.423	.426	2	28.0	3	.257

bryo within the cuticle is unaffected by the following treatment: immersion of the eggs in 70 percent alcohol (15) for 1 hour; washing the eggs with 0.1 N HCl; and floating the eggs in saturated solutions of NaCl or Na_2SO_4. In addition, this cuticle prevents the entrance of radioactive elements even though the egg may be immersed in such solution as P^{32} or Sr^{89} as long as 24 hours.

The effect of β rays on respiration. — In the developing prediapause grasshopper embryo, the oxygen uptake was somewhat affected while the egg was being irradiated. In most cases there was a slight increase in respiration though this increase was not significant (table 1). With very low dosages (2.25 μc per cc) the increase in respiration was greater (table 2). When the embryos were irradiated first and the oxygen uptake measured immediately after irradiation, there was an inhibition of 10 to 35 percent (table 3). Thus, while respiration increased when the eggs were being irradiated with dosages of low intensity, respiration diminished when measured after irradiation.

Barron and Goldinger (16) and Barron *et al.* (17) found that sulfhydryl reagents, when used in low concentrations, cause an increase in oxygen uptake of sea urchin sperm. Barron *et al.* (18) found that nitrogen mustards in low concentrations also cause an increase in oxygen uptake in *Arbacia punctulata* sperm. The effect of nitrogen mustards on gene mutations (19) and tissue resembles the effect of ionizing irradiations. It is possible that the increase of respiration observed when the eggs were irradiated with low dosages of β rays is similar to the effects observed by Barron and co-workers with low concentrations of -SH reagents.

TABLE 2.—HOURLY AND CUMULATIVE RESPIRATION WHILE UNDER ACTIVE β IRRADIATION

Age in days of prediapause grasshopper eggs	Time (hrs)	Average Q^{02} controls		Average Q^{02} irradiated eggs		μc P^{32} (per vessel)	Cumulative dosage (r equivalents)
		cu mm/egg/time indicated	cu mm/egg/hr	cu mm/egg/time indicated	cu mm/egg/hr		
15	1	.292	.292	.349	.349	2.25	3.07
	2	.604	.302	.767	.383	2.25	6.14
	3	----------	----------	1.109	370	2.25	9.21
14	1	.375	.375	.378	.378	21.6	29.5
	2	.769	.385	.763	.382	21.6	59.0
14	1	.358	.358	.406	.406	173	243
	2½	.930	.372	1.002	.401	173	486
	3	1.090	.363	1.168	.389	173	729
	4	1.406	.351	1.491	.373	173	1072
	5	1.766	.353	1.857	.371	173	1215

TABLE 3.—INHIBITION IN RESPIRATION OF GRASSHOPPER EMBRYOS FOLLOWING β IRRADIATION WITH Sr^{89} SOURCE, 70 μC/VESSEL (APPROXIMATELY 100 R GIVEN IN 1 HR)

Age in days of prediapause eggs	Av. Q^{02}		Percent inhibition	Av. Q^{02} cu mm/egg/hr Sr^{89}	Percent inhibition	Percent inhibition due to radioactivity (Sr^{89} less $SrCl_2$)
	cu mm/egg/hr controls	cu mm/egg/hr $SrCl_2$				
9	.322	.285	11.5	.242	24.9	13.4
10	.267	.228	14.6	.198	25.9	11.3
11	.325	.289	11.1	.196	39.7	28.6
11	.271	.269	0.7	.173	36.2	35.5
12	.326	.308	5.5	.206	33.4	27.9
13	.296	.242	18.2	.179	39.5	21.3
13	.198	.196	1.0	.154	22.2	21.2
14	274	.242	11.7	.214	21.9	10.2

TABLE 4.—INHIBITION IN RESPIRATION OF GRASSHOPPER EMBRYOS FOLLOWING β IRRADIATION WITH Sr^{89} SOURCE 2930 μC/VESSEL (APPROXIMATELY 70 R GIVEN IN 1 MINUTE)

Age in days of prediapause eggs	Av. Q^{02}		Percent inhibition	Av. Q^{02} cu mm/egg/hr Sr^{89}	Percent inhibition	Percent inhibition due to radioactivity (Sr^{89} less $SrCl_2$)
	cu mm/egg/hr controls	cu mm/egg/hr $SrCl_2$				
12	.212	.208	1.9	.152	28.3	26.4
13	.299	.271	9.36	.161	46.15	36.8

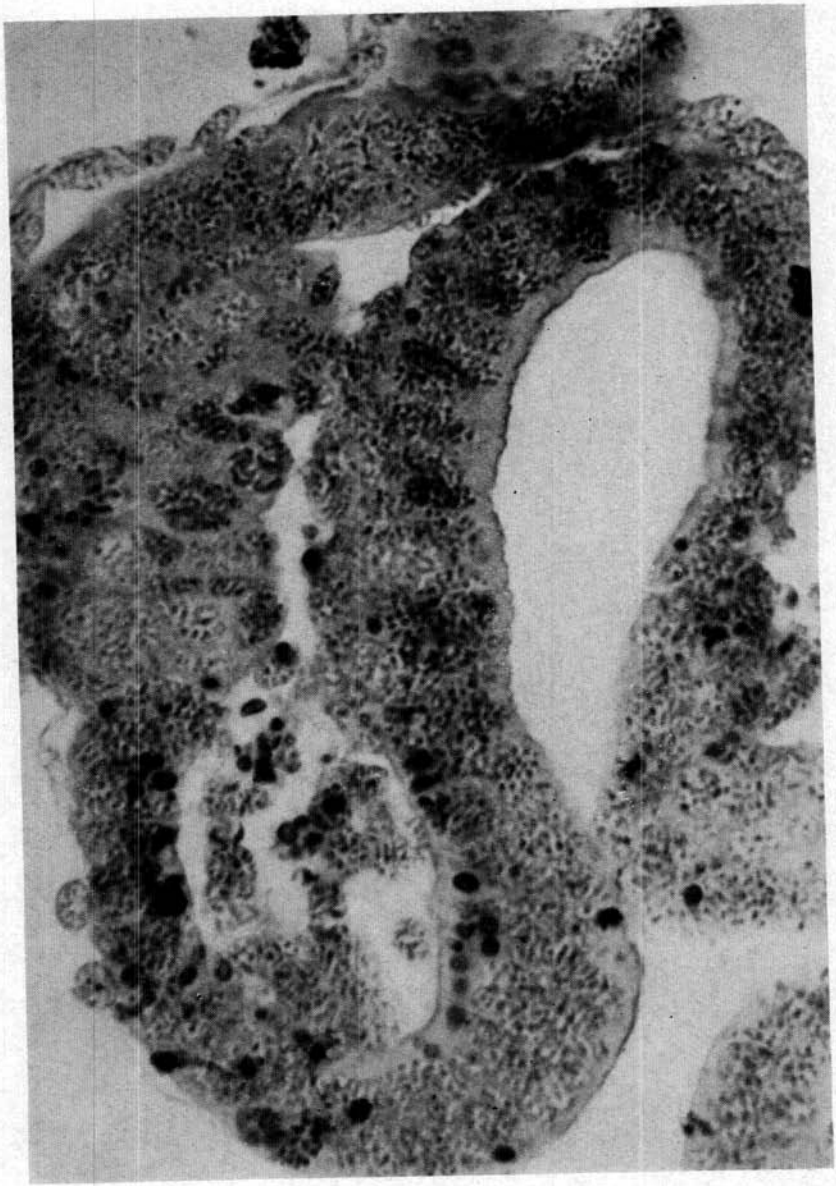

FIG. 1.—Section of an 11-day prediapause embryo showing extensive damage. Total β irradiation, 8 r (2.33 μc P^{32}/cc—2½ hours).

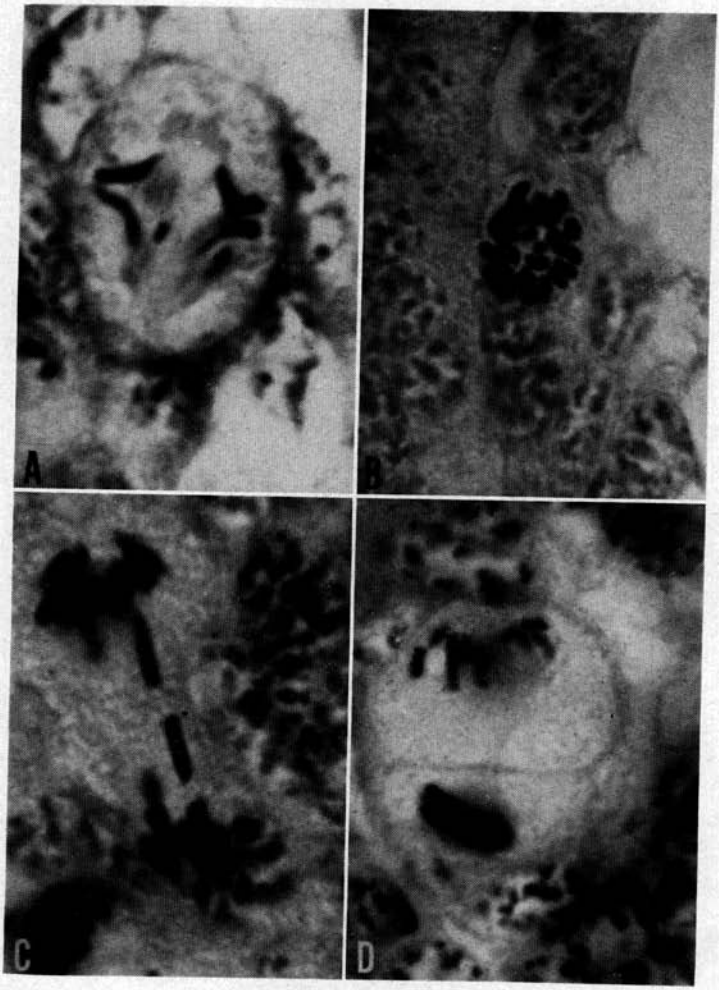

FIG. 2.—A. Normal early anaphase from 11-day prediapause grasshopper embryo irradiated with 8 r (2.33 μc P^{32}—2½ hours). B. Normal metaphase, polar view, from 11-day prediapause embryo treated as in A. C. Late anaphase showing chromosomal break. Other specifications same as A. D. Differential effect of ionizing irradiations on sister nuclei. Cells showing relatively unaffected neuroblast above with pyknosis of the nucleus below; 16-day prediapause embryo, 76.8 r (2.33 μc P^{32}—24 hours).

Morphological alterations produced by β rays.—In figure 2A the extensive damage caused by 2.33 μc of P^{32} in 2½ hours is shown. β irradiation extended over this period is only 8 r equivalents; yet, the abundant pyknosis is evident.

The normal anaphase and metaphase chromosomes appear in figures 2A and B, respectively. These photomicrographs were made from irradiated material. While other nuclei in the same embryo were very pyknotic, these were normal, indicating that the same dosage does not equally affect all the nuclei in mitosis.

In the same embryo we found injury as shown in figure 2D. In figure 2C the cell is extremely elongated. The chromosomes stained red as if in metaphase. One pair of chromosomes is broken so that each sister counterpart is equal in size. The effect must have been caused either in prophase or during metaphase while the chromosomes were in close proximity to each other.

Figure 2D illustrates unequal division resulting in two newly forming nuclei. In the lower half the chromatin is in a very pyknotic stage, while in the upper half chromosomes in late telophase (which normally would be slightly clumped) are segregated. In this cell the division was not complete, as the figure suggests. The apparent division line in the center was caused by focusing the microscope to the most optimal plane where both sides could be photographed. The interzonal fibers are present without any indication of astral fibers. In the upper half a vacuole appears, and the chromosomes are separated into distinct groups.

Varying the exposure of the same negative produced figures 3A and B. In this cell all the chromosomes are abnormal. At the left the chromatin material forms two spheres, while at the right the chromosomes have formed a straight line by cohesion. These have a spindle about them which apparently is functionally ineffective. The spindle is displaced dextrad. In figure 3A the red-staining pyknotic sphere has a blue-staining crescent about it.

Although most of the pyknotic chromosomes stained red, sometimes there appeared a blue-staining counterpart which invariably was separated from the red-staining portion. This suggests that in some instances there might be a reversion of a portion of chromatin material to the prophase component.

Figure 3C shows a cell in which the chromatin is segregated into five groups. Peripherally the clumps stained red, while the centrally located sphere was blue. In figure 3D pyknotic nuclei of three cells stained red without any indication of a blue counterpart.

Figures 4A and B are photomicrographs of the same cell showing the mode of chromosome division, as well as the astrosphere. The chromatin in this cell stained red and is pyknotic. The cell was undergoing division at the time of fixation. This shows that a pyknotic nucleus may divide and with it even the cell may divide; yet, there is a complete lack of interzonal fibers with no deleterious effect on the aster.

Again in figures 4C and D there are two views of the same cell. In this case the effect was not only on the chromosomes, but also on the

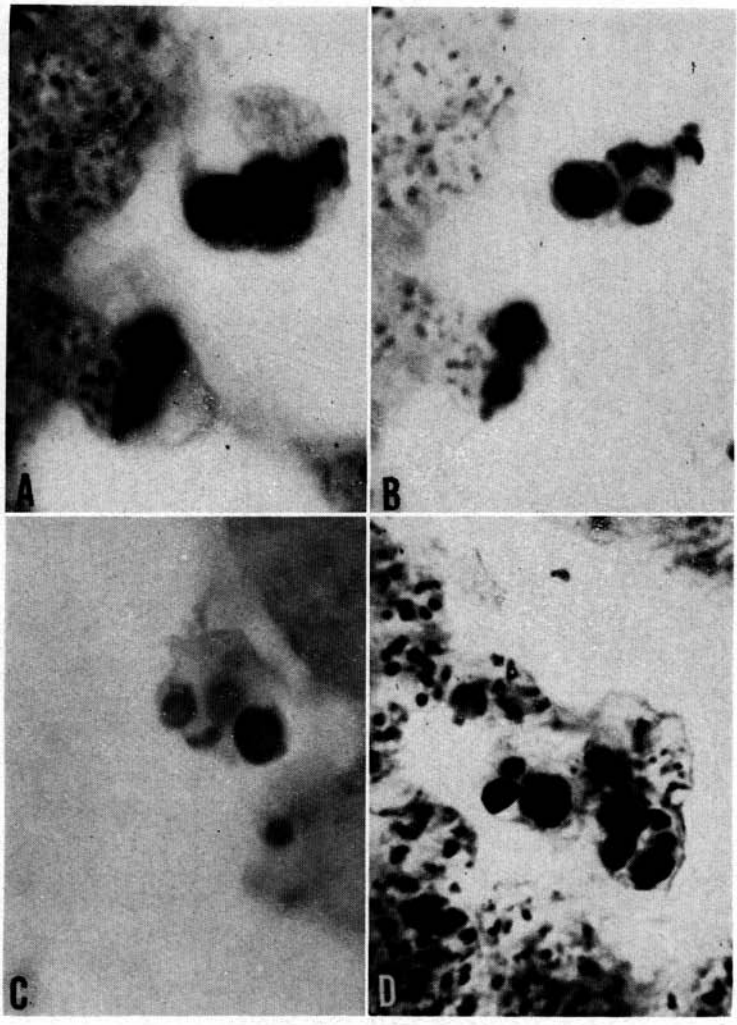

FIG. 3.—A and B. Cells showing partial pyknosis of chromatin with segregation of red and blue staining components. The crescent about the sphere stains blue. The spindle about the injured chromosomes is displaced to the right; 11-day prediapause embryo, 8 r (2.33 μc P^{32}—2½ hours). C. Cells showing pyknotic chromosome with segregation of blue and red staining chromatin material; 15-day prediapause embryo irradiated with 8 r (2.33 μc P^{32}—2½ hours). D. Three pyknotic nuclei without segregation. The damaged chromatin material stains red; 15-day prediapause embryo, 60.8 r (2.33 μc P^{32}—19 hours).

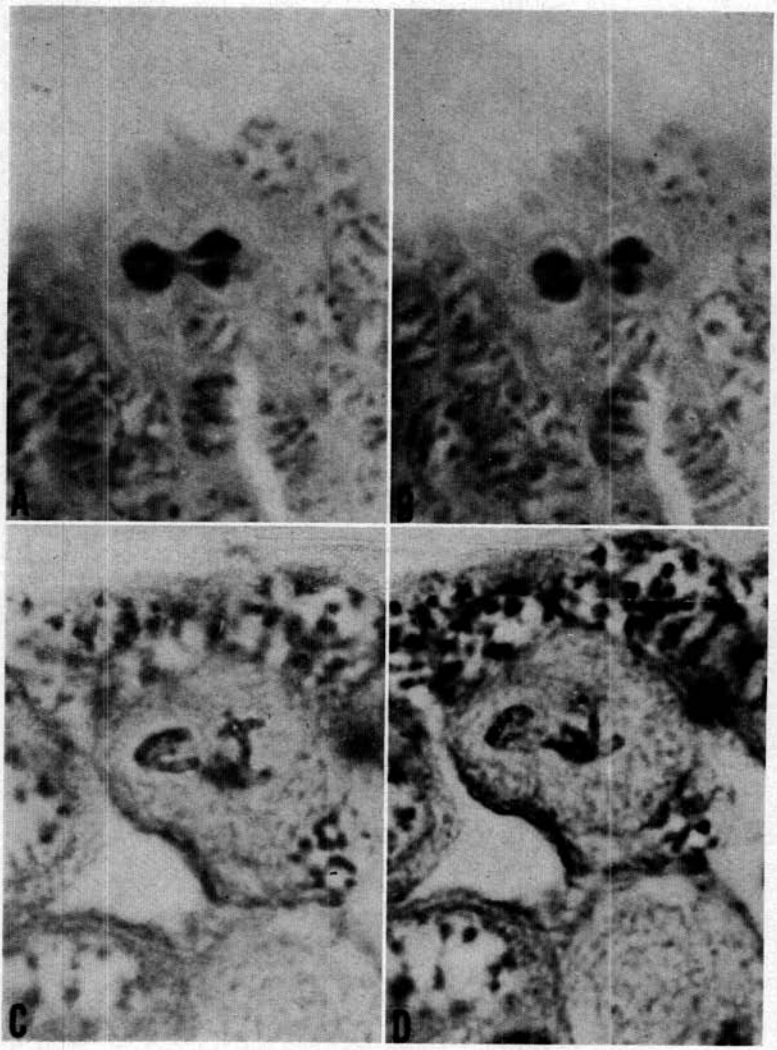

FIG. 4.—A and B. Cell showing pyknotic chromosome in telophase. Mitotic spindle unaffected. Cell division mechanism unaffected; 16-day prediapause embryo irradiated with 60.8 r (2.33 μc P^{32}—19 hours). C and D. Cell showing injured chromosomes, partial pyknosis and cross-over. Osmophilic substance within the chromosome. Astrosphere is displaced 90°. Cell division inhibited; 16-day prediapause embryo, 8 r (2.33 μc P^{32}—2½ hours).

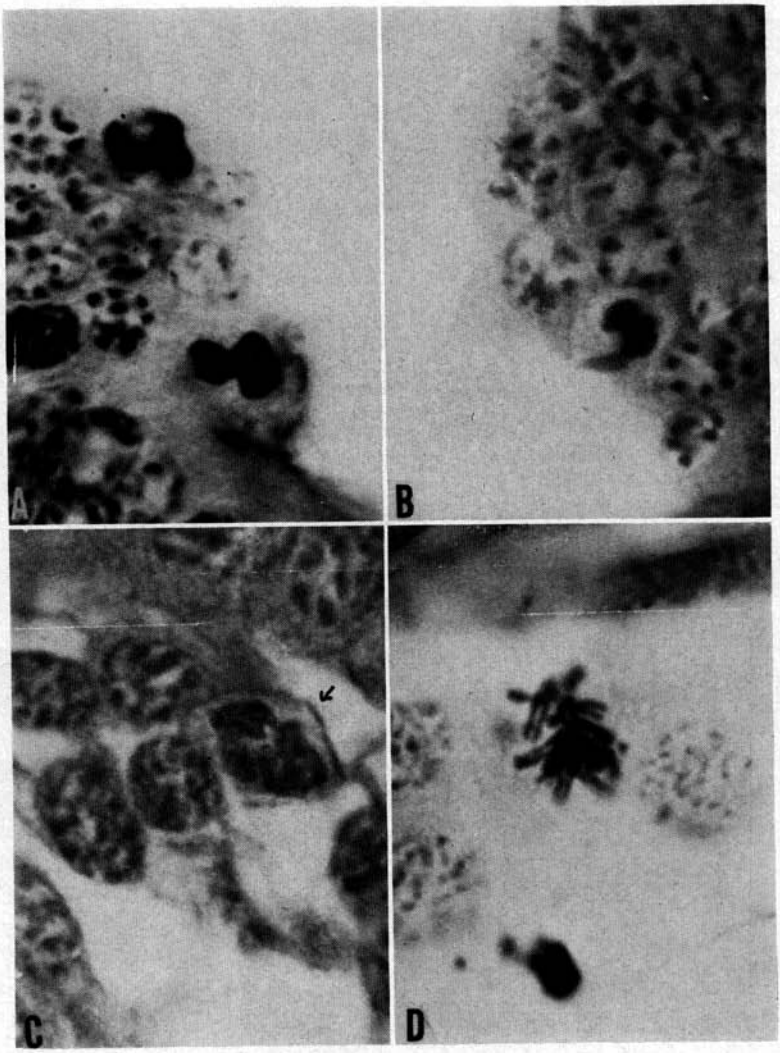

FIG. 5.—A. Cells showing pyknotic nuclei, probably in late anaphase. Mitotic spindle destroyed. Cell not dividing; 15-day prediapause embryo, 60.8 r (2.33 μc P^{32}—19 hours). B. Injury same as A; 16-day prediapause embryo, 8 r (2.33 μc P^{32}—2½ hours). C. Cell showing chromatin material in prophase staining red due to damage. Extreme enlargement of chromosomes. Probable disorganization and incapacity to form the metaphase plate; 15-day prediapause embryo, 60.8 r (2.33 μc P^{32}—19 hours). D. Cell showing chromosomes in prophase with injured telomeres of sister chromosomes; 16-day prediapause embryo, 60.8 r (2.33 μc P^{32}—19 hours).

astral system. There was a monaster, but it was functionally ineffective as the chromosomes were completely disoriented. The chromatin material stained red and within the chromosomes an osmophilic substance was noticeable.

The anastrous conditions are shown in figures 5A-D. In figures 5A and B the pyknotic chromatin material is undergoing division, while in figure 5C the chromosome shows elongation or appears to have united to form a single large chromosome. In figure 5D the chromosomes are distinct, but all the sister chromosomes are united at one end or at the telomere(20). There is complete lack of orientation. These chromosomes stained red.

Figure 6A resembles figure 5C; there is an anastrous condition, but no apparent division of the cell.

Anastrous division, as well as the osmophilic substance, is shown in figure 6B. This indicates that the effect of irradiation can be on the chromosomes and on the mitotic spindle, but not on the process of cell division. Such a case was produced experimentally by Fankhauser(21). He was able to remove the nucleus from the frog's egg, which underwent division without benefit of the aster. In appearance the cell in figure 6B is very much like Fankhauser's drawing.

Figure 6C resembles figure 2D except that the osmophilic material is more apparent.

Figure 6C shows injury from β irradiation where the cell kept growing without division and the prophase chromosomes kept elongating; hence, the injury in this case appears to be on the size-regulating mechanism.

Figures 7, 8, and 9 were included to show the extent of the osmophilic substance associated with the chromatin material and the mechanism of pyknosis.

Figure 7A appears to include a metaphase equatorial plate that has become pyknotic. There is evidence that associated with each chromosome is an osmophilic substance caused by irradiation.

The extent of damage to the chromosomes is seen in figure 7B, and again the osmophilic substance, apparently derived from individual chromosomes, shows up. This condition appears only in pyknotic chromatin material or in chromosomes injured by β irradiation. H_2O_2 will bleach it completely.

A late stage of pyknosis, with small black spheres within the pyknotic red-staining chromatin material, can be seen in figure 7C. This seems to be the outcome of unequal division of the chromatin substance.

Figure 7D contains a cell with a pyknotic nucleus. The red-staining portion contains the osmophilic substance, while the blue-staining portion shows no traces of it. Figure 8A shows a pyknotic nucleus in which a large amount of osmophilic substance is present. A lagging chromosome shows this black substance in the core of the chromosome.

Modes of pyknosis, as well as the appearance of the osmophilic substance, are shown in figures 8B and C. In figure 8D the osmophilic substance is like a spiral throughout the red-staining chromatin. Usually in a pyknotic nucleus that has become spherical, the osmophilic mass is in the form of small spherules, as shown in figures 9A-D. The light spots in the dark-stained portion are caused

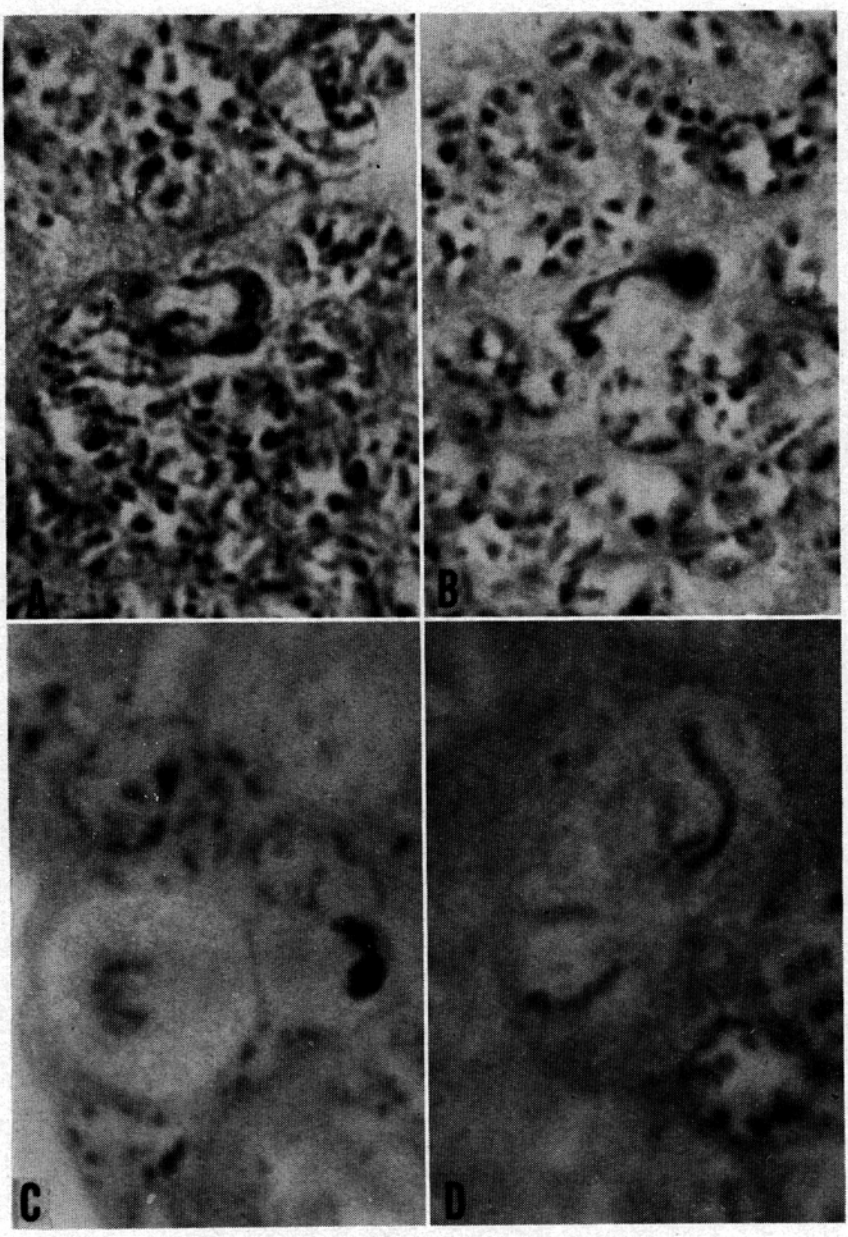

FIG. 6.—A. Cell showing injury to chromosomes and the mitotic spindle as well as the capacity of the cell to divide; 16-day prediapause embryo, 8 r (2.33 μc P^{32}—2½ hours). B. Cell showing anastrous division of pyknotic chromosome containing osmophilic substance. Other specifications same as A. C. Injury to nuclei as shown in figure 2D, except that this is an 11-day prediapause embryo, 8 r (2.33 μc P^{32}—2½ hours). D. Cell showing prophase chromosomes extremely elongated, probably fusion of telomeres between 2 pairs of sister chromosomes; 11-day prediapause embryo, 8 r (2.33 μc P^{32}—2½ hours).

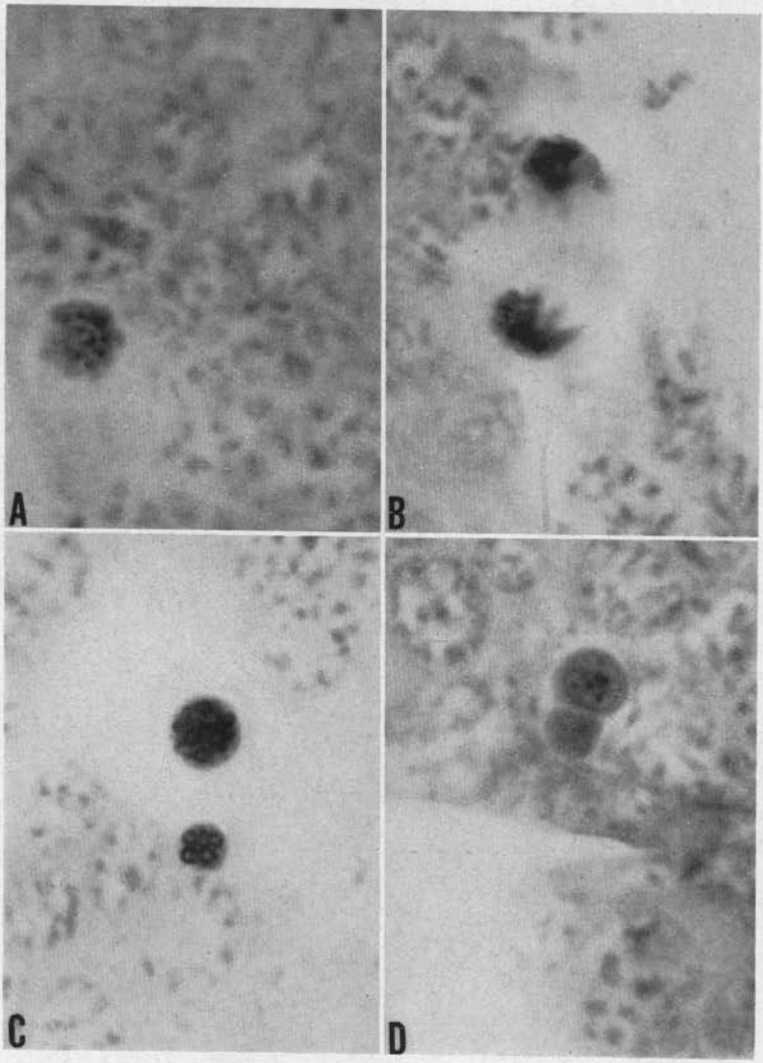

FIG. 7.—From 16-day prediapause embryos irradiated with 76.8 r (2.33 μc P^{32}—24 hours). Photomicrographs indicate mode of pyknosis and association of pyknotic material with chromosomes. Since the pyknotic material is associated with the red staining counterpart, none is found in the blue segregate as shown in D.

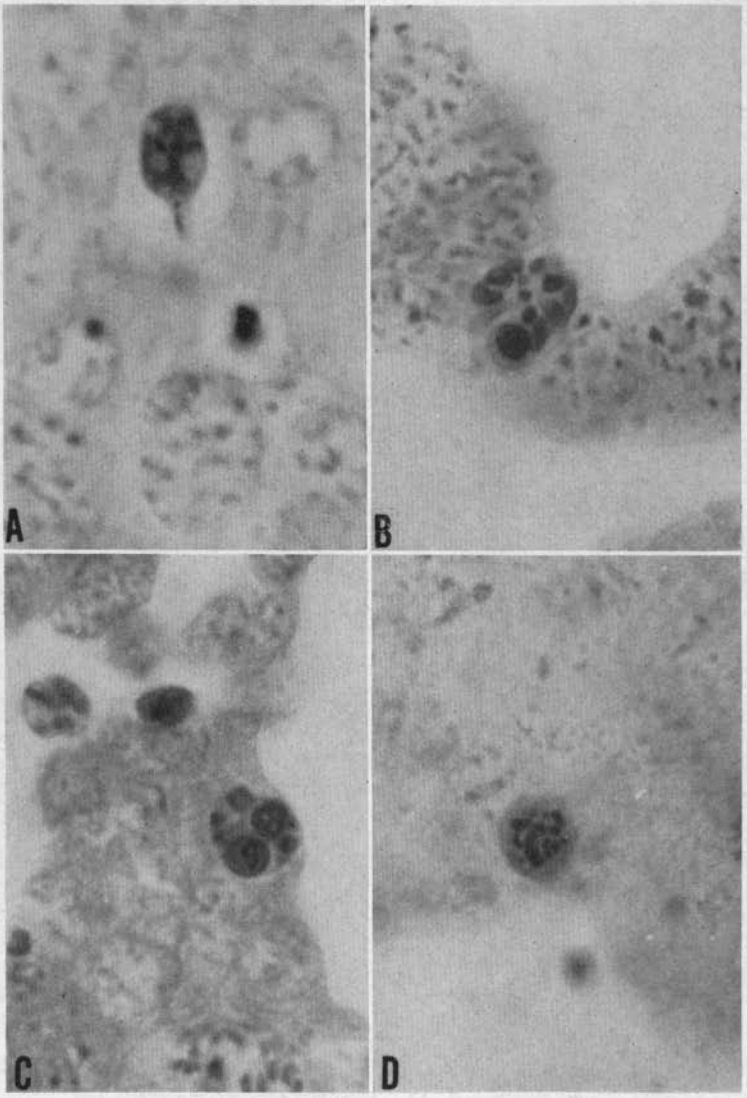

FIG. 8.—Mechanics of pyknosis and association of osmophilic material with chromosomes; 15-day prediapause embryos, 60.8 r (2.33 μc P^{32}—19 hours).

by light refraction when the objective of the microscope is focused below the equatorial plane of the spherule. This demonstrates a vacuous spot caused by refraction about an opaque object.

When X- and γ-irradiated materials were studied, there was no treatment with osmic acid. However, the very light portions seen in the pyknotic nuclei when the Feulgen reaction was used might well be osmophilic components caused by irradiation.

Discussion

The increase in respiration observed when the eggs of grasshoppers were being irradiated with low dosages of β rays (table 1) closely resembles the experiments of Barron and co-workers on the effect of low concentrations of sulfhydryl reagents. Sulfhydryl reagents, which in high concentrations inhibit respiration by inhibition of sulfhydryl enzymes, were found to increase respiration when their concentration was sufficiently diminished. Barron explained these opposing effects by assuming that there are in the cells two kinds of sulfhydryl groups: (1) soluble sulfhydryl groups distributed throughout the cells, which act as one of the regulating mechanisms of cell respiration, and (2) fixed sulfhydryl groups present in the protein moiety of enzymes. Abolition of the first would increase respiration, while abolition of the second would inhibit it (tables 1, 2, 3, and 4). Perhaps when the cells were being irradiated with low dosages of β rays, there was oxidation of the soluble sulfhydryl enzymes with subsequent rupture of the regulation of respiration, a phenomenon similar to the increase in respiration observed by Barron *et al.*(17) on addition of small amounts of iodosobenzoic acid (an oxidizing agent) to sea urchin sperm. The inhibition of respiration observed when measurements were made after irradiation is difficult to explain.

The Feulgen reaction has been a very useful tool in discerning the building blocks of chromosomes but, unfortunately, the structure formed by these blocks is as obscure today as it has been in the past. The desoxyribosenucleic acid is associated with histones and protamines(22). Yet when we test for the nucleic acid we are not dealing with the original nucleoprotein complex—we are dealing with the hydrolytic products of the nucleoprotein. Even the desoxyribosenucleic acid itself must be hydrolyzed before any reaction is obtained. Undoubtedly desoxyribosenucleic acid is present in all the mitotic stages regardless of what the new structure happens to be. Hence, in every stage of mitosis we obtain a test for desoxyribosenucleic acid. However, there are investigators who maintain that the only test for the presence of desoxyribosenucleic acid is the treatment of the alcohol-fixed material with thymonucleodepolymerase, after which treatment Feulgen's reaction does not take place(23).

For this study we chose Flemming's triple stain because it opens a new mode of attack on irradiation problems. Even though fixation alters the original structure of the cell constituents, irradiation causes permanent changes that are demonstrated with the use of this stain.

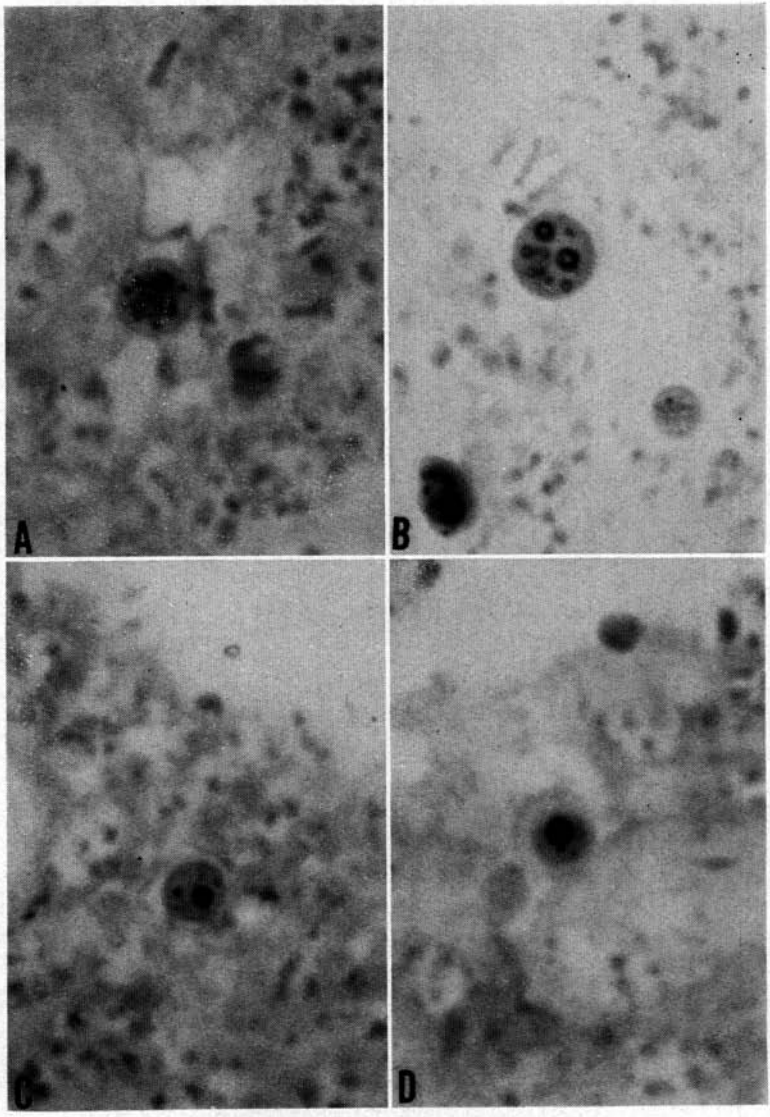

FIG. 9.—Osmophilic substance within completely pyknotic nuclei from embryos irradiated with 8 r and 60.8 r.

We have already mentioned that normally the stages of mitosis demonstrate a differential affinity to these dyes, which indicates the normal structural changes of chromosomes. β irradiation altered the structure so that injured chromosomes and pyknotic nuclei always stained as if the chromosomes were in the metaphase state.

Evidence has been found that sister cells in the same morphological stage are affected differently with a given dosage of β irradiation (see figures 2D and 6C). These cells that are being formed are undergoing primary differentiation. The larger cell above is a neuroblast formed by delamination from the ventral ectoderm(25). The tissue into which each has differentiated can be an attribute of the specific gene introducing its potency in one cell while this attribute of the sister gene in the other cell is repressed. In order to enter primary differentiation the effect must be on the whole cell. The whole cell, owing to this differentiation and irrespective of the mitotic state, has become more susceptible to the ionizing irradiation. Injury in these cells is probably sustained by both sister cells, but the degree of injury is different; one may recover while the other will become pyknotic.

Figures 4C and D (different views of the same cell) show that injury was sustained not only by the chromosomes but by the mitotic spindle as well. In this cell the spindle is displaced 90° so that one aster is below the chromosomal mass. In a previous communication(11) we found that the effects of irradiation of the magnitude used here are completely reversible, or else the injured portion is phagocytized(20).

Division of pyknotic chromatin material as seen in figures 4A and B can hardly be equal in each sister cell. If the chromatin material were to rearrange itself later, the gene components on each side would be different. This might cause a lethal effect in one or both, not because of gene injury, but because of lack of the gene due to unequal chromosomal division.

In this series of irradiated embryos we have noticed a new phenomenon because the translucent condition obtained in chromosomes by the use of Flemming's triple stain made it possible to examine the interior of these structures. Wherever there is a pyknotic nucleus or an injured chromosome there is usually some black-staining material within the chromatin which is apparently an osmophilic substance. With increased dosages of radiation more of these black spheres appear. Since the procedure in fixation necessitated treatment with osmic acid, we bleached some of the prepared slides in H_2O_2 and found that the black-appearing spheres and granules disappeared. Soaking the slides in benzene or toluene overnight did not bleach out this black material. This artifact, whatever it is, is due to irradiation. We want to carry out further experiments before making more definite statements concerning this finding, but we are convinced that this material is derived from individual chromosomes since not one normal-appearing chromosome contained the black material. Osmic acid stains the unsaturated fatty acids and fat soluble materials.

The presence of this material may be caused by dehydrogenation of saturated fatty acids by ionizing irradiations.

Pyknosis of chromosomes produced by ionizing radiations may be due to the following injuries: (1) to the structure of the chromosomes, (2) to the mechanics of cell division, (3) to the mechanics of cell division and the chromosomes, (4) to the gene or telomere, (5) to the nucleus because of the lack of a gene, and (6) to the cell, owing to the differentiation of the cell caused by the unfolding of a gene.

Summary

Low dosages of β radiation produced extensive damage in the embryonic cells of grasshoppers. The respiration of grasshopper embryos was increased during irradiation; immediately after irradiation there was inhibition. By the use of Flemming's triple stain it was shown that β irradiation caused pyknotic chromatin to remain as if it were in the metaphase condition. There is evidence that injury sustained by the chromosomes may be direct and indirect. β irradiation caused the appearance of some osmophilic material associated with the chromosomes. The nature of this material is as yet unknown.

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