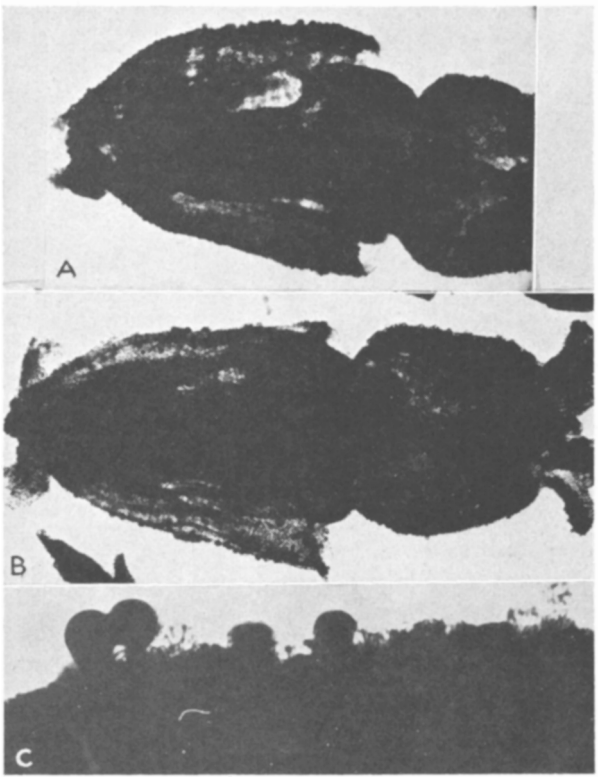

FIG. 2. Two different squids. Age 2 days. Kept for 20 minutes in sea water with a content of .005% neutral red. The surface of the skin is covered with smaller and larger droplets of dark red colored sweat. Enlargement 25 ×. *Lowest figure,* a part of the apical (aboral part of the body) of a squid eliminating droplets stained with neutral red from its skin. (Enlargement 250 ×). Four such droplets well recognizable, growing more and more to a bowl shape. If this stage is reached, slight movement of the animal may separate the droplets from the body.

A Phase Contrast Microscopy Study of X-Ray-Induced Pyknosis in the Living Cell. (19153)

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(Introduced by Douglas E. Smith.)

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This study was undertaken to determine the cytological effects of X radiation on the living cell. X-ray-induced pyknosis, as seen in fixed preparations, has been discussed in a previous communication(1); however, the effects of fixatives upon these cells were not determined. Therefore, with the aid of the phase contrast microscope, an attempt has been made to determine whether fixatives alter the cytological pattern of irradiated cells. Although the cytological pattern observed in living and fixed preparations appears to be similar, the physical state of the cell components varies in the living material.

Materials and methods. Melanoplus differentialis egg pods were collected daily from an indoor colony and stored on moist sand at a constant temperature of 25° ± 0.02°C for 30

1. Tahmisian, T. N., and Gasvoda, J., *Quart. Rep., Biol. and Med. Divisions, Argonne National Laboratory,* Aug., Sept., Oct., 1948, eds., A. M. Brues and H. Lisco. ANL-4227, p154-180.

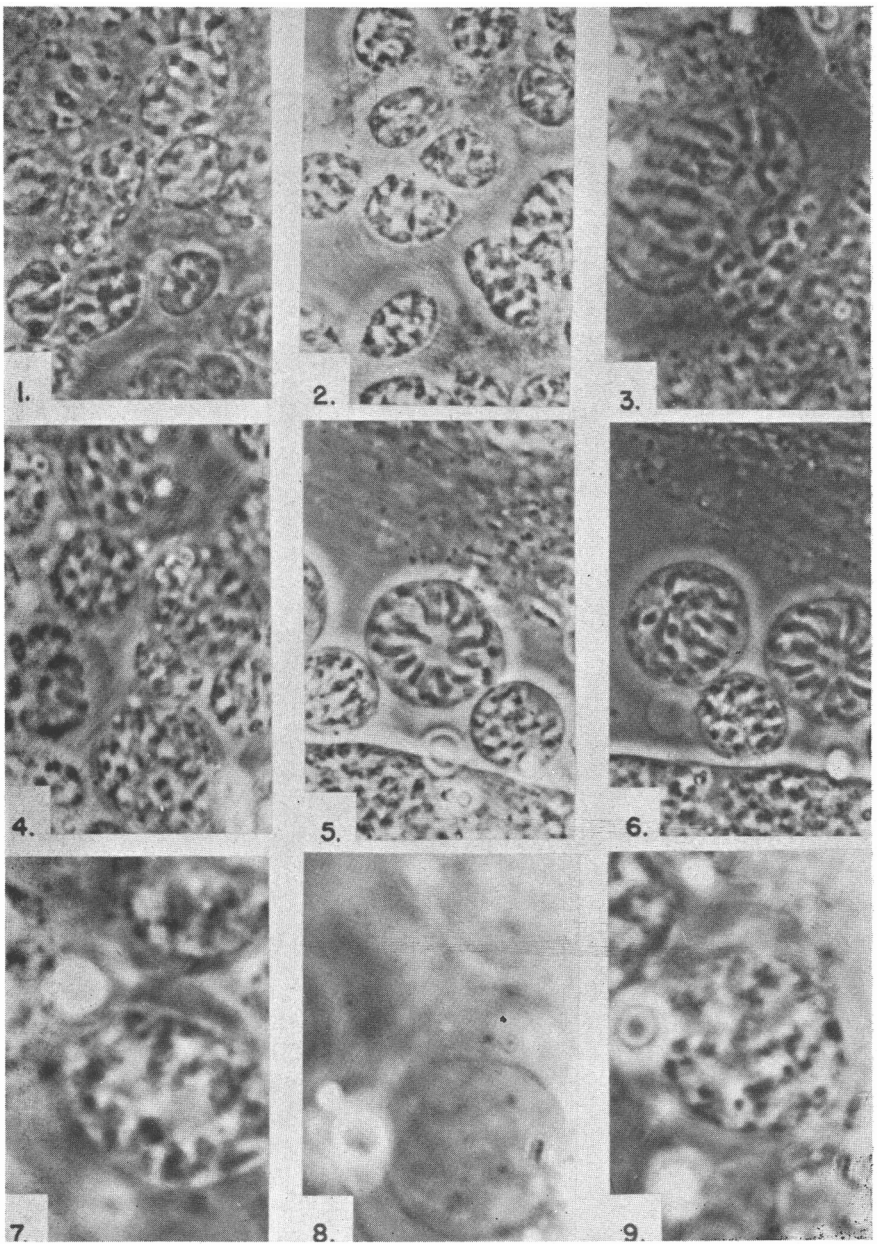

Plate 1. *Melanoplus differentialis* embryo cells. 1120 ×.

FIG. 1. Control, one hr postdiapause.

FIG. 2. Cells X-irradiated with 25000 r 6 mo. previously, kept at 2° ± 1°C. One hr after removal from low temp. 1120 ×.

FIG. 3. Early prophase control, 24 hr postdiapause. 1520 ×.

FIG. 4. Late prophase control, 48 hr postdiapause. 1120 ×.

FIG. 5. Metaphase, polar view, control 96 hr postdiapause. 1120 ×.

FIG. 6. Early anaphase and polar view metaphase, 96 hr postdiapause. 1120 ×.

FIG. 7, 8, and 9. Photographs of the same interphase cell. 1680 ×. (7) In modified Belar sol.; (8) In distilled water; (9) In modified Belar sol. after being placed in distilled water.

days. The eggs were then removed from the pods, washed, sterilized, and dechorionated with 2.5% NaOCl(2). They were rinsed in several changes of double glass-distilled, pyro-

gen-free water, treated with 0.2% citric acid to neutralize the NaOCl, and rinsed again with distilled water. A Spencer stereo-microscope was used to select the experimental material according to the following criteria: 1) the presence of an embryo, 2) the presence of a cuticle, and 3) an intact amnion which indicated that the egg was in diapause. The selected eggs were divided into two groups; one kept as the control and the other X-irradiated at 25,000 r (dose rate, 1433 r/minute; filter, pyrex petri dish cover; 200 kv; and 15 ma). The irradiated material was divided into two portions, one of which, with appropriate controls, was kept at 25° ± 0.02°C, and the other, with controls, at 2° ± 1°C for 6 months. The embryos were dissected in the following modified Belar solution with final concentrations of 0.9% NaCl, 0.05% KCl, 0.05% $CaCl_2$, and 0.01 M KH_2PO_4 + Na_2HPO_4, pH 6.8. The photographs were taken with the aid of a Leitz ortholux microscope that was modified with American Optical Co. phase contrast lenses and a phase condenser. The American Optical Co. dark M phase contrast, 97 X oil immersion with 1.25 N.A., was used. The substage condenser was lowered until a green yellow ring was observed through the telescope, and the pictures were taken on Eastman Kodak 35-mm panatomic X film at a spectral band of 5000 Å to 5800 Å units without other filters. Brownian movement was studied and photographed with the aid of an Eastman Ciné Kodak Special camera at 16 frames per second. The effect of X radiation on the mitochondria of living cells was determined cytochemically with Janus green, (certified vital stain No. D274, City Chemical Corp., N. Y.), 1:50,000 parts in modified Belar solution. The modified Belar solution was used because cells in prophase completed mitosis indicating this solution was not injurious to cells. The effects of Belar, refractive index, 1.3339; distilled water, refractive index, 1.3330; 10% sucrose, refractive index, 1.3480; 5% dextrose, refractive index, 1.4020; buffer (pH 3.40-9.80) at 20°C as determined with the aid of an Abbé Bausch Lomb refractometer; and CH_3COOH and NH_4OH, on the prediapause, diapause, and postdiapause nuclear chromatin were determined (Table I). The condition of chromatin in the nucleus of embryonic cells in their natural fluid and of white cells of undiluted human blood was observed.

Results. In diapause grasshopper eggs irradiation-induced physiological and cytologicals changes can be kept latent for 6 months by maintaining the embryos at 2° ± 1°C. In nature cold treatment is a normal event that breaks the diapause block, and this condition is fulfilled by the onset of winter. If the diapause eggs are not cold-treated for at least one month, no further development takes place. When control embryos are removed from 2° ± 1°C to 25° ± 0.02°C and observed for cytological and morphological changes, they are found to resemble diapause embryos, Fig. 1; however, 24 hours after being placed at 25°C, mitotic figures appear. After 96 hours at this temperature many cells undergo division (Fig. 5 and 6). Eggs that received 25,000 r before a six-month cold treatment period (Fig. 2) showed no irradiation effects during the first three days at 25°C when examined with a phase contrast microscope. On the fourth day irradiation injury became apparent (Plate 2). Of the 379 embryos studied not one normal mitotic figure was found in the irradiated embryos over a period of 18 days at 25°C. Irradiation injury began with a pseudoprophase (Fig. 10-12). The chromosomes became larger and the nuclear membrane became more apparent, but remained intact. As the chromatin showed signs of pyknosis, the embryo began to undergo negative growth(3). At the same time there was an increase in an oxidative system that oxidized hydroquinone at a rate that was 400% higher than in the nonirradiated egg (4). The nucleus in many cells showed an increase in Brownian movement, suggesting that liquefaction was taking place; in others, no motion was apparent indicating gelation. Although the pyknotic bodies appeared morphologically the same in fixed and living material they were physiologically in various

2. Slifer, E. H., *Science*, 1945, v102, 282.

3. Tahmisian, T. N., *J. Exp. Zool.*, 1949, v112, 449.

4. Tahmisian, T. N., and Adamson, D. M., in press.

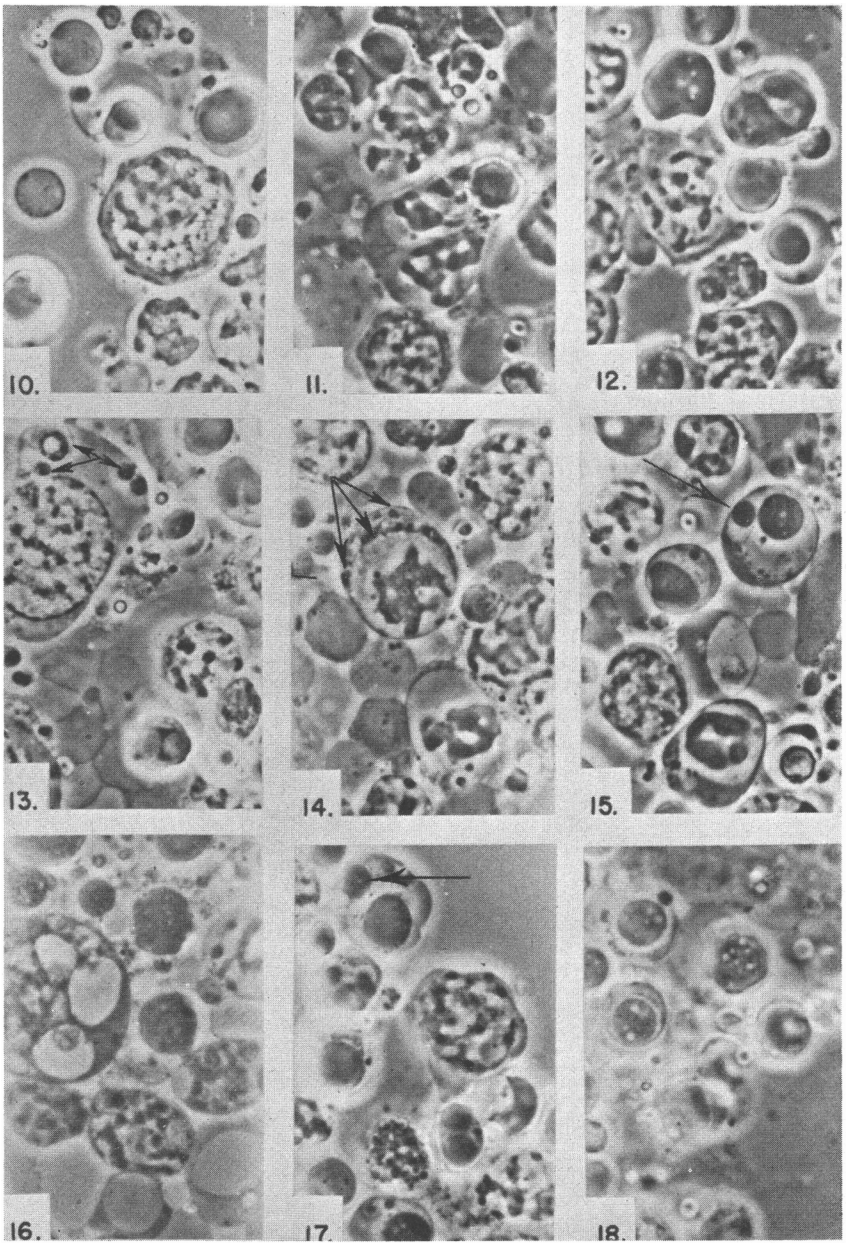

Plate 2. *Melanoplus differentialis* embryo cells cold-treated, 2° ± 1°C, for 6 mo. after X-irradiation with 25000 r, then removed to 25° ± 0.02°C.

FIG. 10. Ninety-six hours after cold treatment, nuclear membrane enlarged, chromosomes sticking to nuclear membrane as well as to one another. 1120 ×.

FIG. 11. Six days after cold treatment. A type of pseudoprophase in the sequence of pyknosis. 1120 ×.

FIG. 12. Six days after cold treatment pyknotic and injured nuclei. 1120 ×.

FIG. 13. Eight days after cold treatment. Enlarged nucleus showing incorporation of nuclear membrane with chromatin, also clumping of mitochondria into 4 clusters in cytoplasm. 1120 ×.

FIG. 14. Ten days after cold treatment. Pyknotic chromatin material and large mitochondria at periphery of nuclear membrane. Early segregation of lipoids from the chromatin mass. 1120 ×.

FIG. 15. Twelve days after cold treatment. Lipoid segregation in pyknotic nucleus and absence of lipoid material in pyknotic mitochondrial mass as tested with Janus green. 1120 ×.

FIG. 16, 17, and 18. Eighteen days after cold treatment showing extensive pyknosis and diminution in cell and nuclear size. 1120 ×.

TABLE I.

pH*	Solution	Chromatin	2 min.	On addition of	
3.40 to 9.80	PO_4 Belar	visible	visible	H_2O	invisible
10.50	NH_4OH Belar‡	invisible	invisible	CH_3COOH	visible
6.60	10% sucrose‡	visible	"	Belar	"
6.52	5% dextrose‡	"	"	"	"
6.2	distilled water‡	"	"	"	"

* 6-9.80 the increment was at intervals of .2 to .5 pH.
† These solutions were not buffered.

phases of gel-sol stage in the living condition.

As pyknosis progressed there was always an increase in the size of mitochondria (Fig. 13 and 14). In irradiated cells mitochondria have a high rate of Brownian movement. The rate of movement in the injured cell was much greater than that found in metaphase of normally dividing nonirradiated cells. The mitochondria eventually clumped into a separate body from several small groups (Fig. 13-15). As the chromatin assumed a spherical shape, due to pyknosis, the mitochondria had a similar appearance (Fig. 15 and 17, note arrows). Within the pyknotic chromosomal counterpart lipoid bodies appeared as clear dots under dark medium phase contrast. When cells were fixed in osmic acid-containing fixatives these globules in the pyknotic chromatin were osmophilic(1). This did not hold true for the mitochondrial pyknotic mass (Fig. 15). Eventually the two pyknotic spheres merged and could no longer be identified as separate entities (Fig. 18).

The effects of electrolytes and nonelectrolytes on the living nuclei are shown in Table I. Chromatin was always distinctly visible in the cells of the normal grasshopper embryo when observed with phase contrast microscope. *In situ* studies of the fly *Sciara* show chromatin in the salivary gland(5). Human leucocytes showed the nuclear pattern in undiluted blood if the blood was observed immediately after withdrawal. In the modified Belar solution the cell has the capacity to complete mitosis from a known prophase through telophase. The resting nuclei in the vicinity of the dividing cells showed the chromatin as a distinct phase within the nuclear membrane. The chromatin in an interphase grasshopper cell was distinct in its embryonic fluid under phase contrast in the living condition. If the embryo was dissected in 10% sucrose or 5% dextrose, chromatin structure in the interphase nucleus was present when observations were made within two minutes after dissection. The chromatin in these resting cells lost its identity as the cells remained in nonelectrolytes. If resting cells were dissected in modified Belar solution, the chromatin was distinct. When distilled water was introduced from the side of the coverslip the structure of the chromatin lost its identity. After all nuclear structure had disappeared, addition of modified Belar solution following the water treatment reestablished chromatin structure. Fig. 7, 8, and 9 are a series of phase contrast photographs of the same cell: Fig. 7, in modified Belar; Fig. 8, Belar to which water has been introduced; and Fig. 9, to which in turn modified Belar has been introduced. The refractive indices of the solutions do not indicate loss in intranuclear structure. In distilled water and in 5% dextrose, having the lowest and highest refractions, respectively, the chromatin lost its identity in time. The extended or contracted form of the chromatin in the resting cell does not indicate normality nor injury. In NH_4OH above pH 9.8 no structure was seen, but at pH 3.5 in CH_3COOH acid, the previously lost structure reappeared. Although in both cases the cells were injured.

Discussion. Chromatin structure in the interphase cell of the grasshopper embryo was visible in physiological solution as well as in its own embryonic fluid when observed with dark M phase contrast equipment. The cell had the capacity to complete division from early prophase through telophase in modified Belar solution. The criterion of no injury to cells, as stated by Ris and Mirsky(6), is the capacity of the cell to complete division.

5. Buck, J. D., and Boche, R. D., *The Collecting Net*, 1938, v13, 201.

We accept this definition and in addition, believe that other cells treated in the same way but which cannot divide will not be injured by the physiological saline in which they are placed. The changes described above in the living cell are attributed by us to the effects of irradiation.

Since a 6-month cold-treatment period following irradiation is known to delay the appearance of injury, changes that were observed in the cell following irradiation are dependent on the metabolic state. If the cell is maintained at developmental temperatures, gross morphological changes take place within a few days following exposure to 25,000 r. This suggests that the cell has lost its capacity to maintain the *status quo*.

Radiation-induced changes consist of an increase in Brownian movement within the cytoplasm as well as in the karyoplasm of most cells. Increase in Brownian movement indicates a drop in the viscosity within the cell following X irradiation at 25,000 r. Other cells, which are relatively few, show increased viscosity as judged by the lack of visible Brownian movement.

The increase in the size of the nucleus and a drop in its viscosity in many cells suggests that a change in the permeability of the nuclear membrane takes place following irradiation. This effect, however, is not immediate but is apparent only after 4 to 8 days following irradiation, when the cell is maintained at metabolic temperatures. In excised nuclei we were not able to detect any osmotic effects upon the membrane. Our method for determining such effects, however, may not be valid since removal of the nucleus from the cytoplasm may have injured it. Irradiated pyknotic nuclei could not be removed from the cytoplasm without the loss of nuclear structure.

The mitochondria in the living, irradiated material showed great Brownian activity and eventually clumped to form clusters in the cells. There seems to be a general rule that the various constituents within a cell that normally are maintained as separate entities do not have the capacity to maintain their spatial relationship for a very long period following X irradiation.

Summary. The effect of X radiation with 25,000 r can be kept latent in *Melanoplus differentialis* embryos for 6 months by maintaining them at submetabolic temperatures. In the resting grasshopper nucleus chromatin, in Belar's saline solution, is visible with the aid of phase contrast microscopy. When the eggs are irradiated and placed immediately at 0°C and kept at this temperature for 6 months no immediate morphological or physiological changes are observed upon returning them to 25°C. However, pyknosis occurs by 8 days at 25°C depending on the irradiation and on the metabolic activity of the cell. Pyknosis in the living cell as observed by phase contrast microscopy resembles fixed material morphologically. Physiologically there is a difference in the physical state of the nuclei when observed in the living condition. Most nuclei show a drop in viscosity at the time of pyknosis as judged by the increase in Brownian movement. Vital staining with Janus green indicates that the mitochondria usually clump as a separate body whereas, the chromatin material becomes pyknotic containing lipoid impregnated bodies.

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6. Ris, H., and Mirsky, A. E., *J. General Physiol.*, 1949, v32, 489.

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