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Influence of Yolk on Mitotic Rate in Untreated and X-Rayed Grasshopper Neuroblasts *in vitro*.* (22018)

MARY ESTHER GAULDEN AND KATHERINE L. KOKOMOOR.
(Introduced by Alexander Hollaender.)
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

The 14-day-old embryo of the grasshopper *Chortophaga viridifasciata* (De Geer) has been extensively used by Carlson, Gaulden, and coworkers for *in vitro* studies of its large, rapidly dividing neuroblasts. In the course of some experiments requiring rapid preparation of hanging-drop cultures of irradiated embryos, the small amount of grasshopper egg yolk usually added to the artificial medium (1) in the cultures was omitted to speed operations. The recovery of the neuroblasts in these cultures from radiation-induced mitotic inhibition was greatly retarded. The present report describes a detailed examination of the influence of yolk on mitotic rate in untreated neuroblasts and on recovery from mitotic inhibition in X-rayed neuroblasts. A positive influence was found in each case.

Materials and methods. In all experiments embryos were dissected and made into hanging-drop preparations for *in vitro* observations as described by Gaulden, Nix, and Moshman (2), with two exceptions. Following separation from their membranes and removal of their appendages (hereafter referred to as "dissection"), the embryos were left surrounded by yolk in a dish of artificial culture medium until they were mounted in hanging-drop cultures. Varying amounts of yolk, or no yolk, were added to the cultures. For study of the influence of yolk on mitotic rate of untreated embryos, 4 different amounts were tested, namely, approximately 0, 1/8, 1/4, and 1/2 the amount present in an egg. Amounts of yolk greater than 1/2 were not used because they increased greatly the osmotic pressure of the culture drop resulting in a change in mitotic rate(3). With the amounts of yolk used in the present experiments, no changes in osmotic pressure—as determined by the visible appearance of cells and chromatin—were observed except possibly during the later counting periods in embryos mounted with 1/2 yolk. A given amount of yolk can be obtained by placing a 14-day-old egg (Fig. 1) in a dish of culture medium,

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cutting off the posterior tip of the egg, and gently pressing the embryo and yolk out of the chorion. If this operation is performed carefully, the yolk comes out in the form of a cylinder with the embryo lying on top at one end. The embryo and its membranes can be easily discarded without disturbing the yolk. The cylinder of yolk can then be cut into portions of desired size. The anterior ⅛, ¼, or ½ of the yolk cylinder was used because its consistency was more uniform than that of the posterior portion. Currents in the medium around the yolk are kept at a minimum during these operations in order to prevent dispersal of the serosal fluid which surrounds the yolk. Twelve minutes after dissection each embryo was mounted on a cover glass, ventral surface down, in a drop of culture medium to which was added a given amount of yolk or no yolk. The total amount of culture material in the hanging-drop was equal to 5-10 μl of fluid. The cover glass was inverted and sealed over a depression slide with thick mineral oil. The volume of the depressions used varied from 0.0531 to 0.0594 ml. Twenty-one minutes after the embryos had been dissected the hanging-drop preparations were placed on the stage of a microscope, the lower portion of which was enclosed in an incubator maintained at 38° ± 1°C. Observations on the neuroblasts, which lie just beneath the cover glass in these preparations and which are in all stages of mitosis, were begun 1 minute later with the oil immersion lens and bright-field illumination. Light from the microscope lamp was passed through a 5% solution of copper sulfate to remove heat. For study of the influence of yolk on recovery of neuroblasts from X-ray-induced mitotic injury, embryos were exposed *in vivo* to 64 r of X rays at room temperature (25° ± 2°C). A General Electric Maxitron 250-kvp unit was used at 125 kvp and 15 ma with 3.5 mm Al filtration and 54.5 cm target distance. The dose rate was 32 r/minute. Embryos were dissected 18 minutes after irradiation and mounted 2 minutes later. In preparation of the hanging-drop cultures, the X-rayed embryos were mounted, half with ¼ yolk and half without yolk. Untreated control embryos were similarly mounted. Twenty-one minutes after irradiation, the hanging-drop cultures of both treated and control embryos were placed in the incubator at 38° ± 1°C and observations begun 1 minute later.

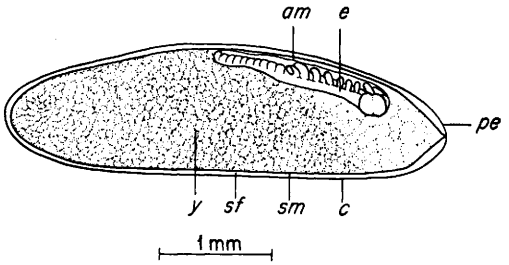

FIG. 1. *Camera lucida* sketch of 14-day-old egg of grasshopper, *Chortophaga viridifasciata*. *am*, amnionic membrane; *c*, chorion and closely adhering cuticle layers; *e*, embryo; *pe*, posterior end of egg; *sf*, serosal fluid; *sm*, serosal membrane; *y*, yolk.

The method for determining mitotic rate is as follows. Approximately 140 neuroblasts per embryo (all neuroblasts in the 3 thoracic segments) are examined at 16 22-minute intervals and the number of neuroblasts in mid-mitosis (prometaphase, metaphase, and anaphase) are recorded. Such counts provide a valid determination of the number of cells going through mitosis in a given period of time because the duration of mid-mitosis at 38°C is 22 minutes under the conditions of the present experiments. The short interval between observation periods limits the number of embryos that can be examined at one time. Thus the data for each experimental condition represent a compilation of data obtained from several sets of embryos studied on several successive days. In the experiments to test the influence of yolk on untreated mitosis, 2 embryos with each of the 3 amounts of yolk and 2 with no yolk were prepared for study at a time. In the X-ray experiments, 4 X-rayed and 4 untreated embryos, 2 with and 2 without yolk in each case, were studied at a time.

Results. Results of the experiments designed to determine the influence of yolk on mitotic rate of untreated neuroblasts are given in Fig. 2 as the average number of mid-mitotic cells per embryo plotted against time after dissection. An analysis of variance test† of

† The authors are indebted to Dr. A. W. Kimball and Mr. George Atta for the statistical analyses of the data.

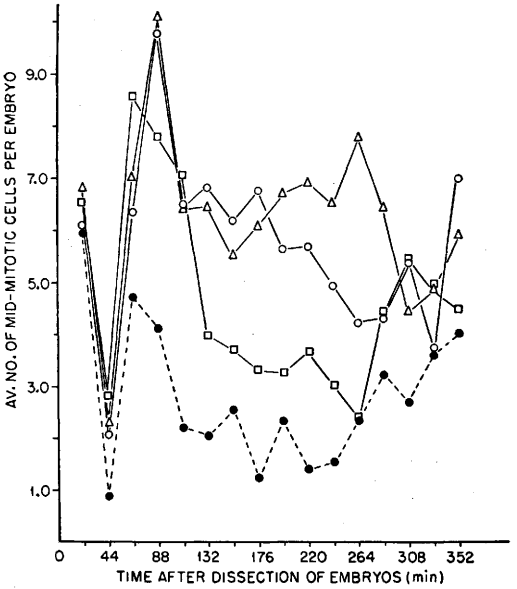

FIG. 2. Effects of varying amounts of yolk from a grasshopper egg on mitotic rate of untreated neuroblasts. ●, no yolk (8 embryos); □, ⅛ yolk (9 embryos); △, ¼ yolk (8 embryos); ○, ½ yolk (7 embryos).

the raw data revealed that the shapes of the curves are essentially the same but that their over-all heights are different at the 0.1% level of significance. The number of mid-mitotic cells in the no-yolk cultures as compared with the yolk cultures at the 44- and 66-minute counts (Fig. 2) was significantly lower only at the 5-10% level of probability. This cannot be considered a statistically significant difference, but it does indicate an early trend toward low mitotic activity in the no-yolk cultures that reaches significant proportions by the 88-minute count. In other words, the decrease in number of cells that can complete mitosis in the absence of nutritive yolk becomes evident soon after the cells are separated from the yolk.

The drop in the frequency of mid-mitotic cells at the 44-minute count followed by a rise at the 66- and/or 88-minute counts occurred in all 4 types of culture preparations. Exploratory experiments revealed that this fall and rise of activity results from a brief inhibition of mitotic progress of cells which enter or are in middle and late prophase when cultures are prepared. This inhibition appears to be caused partly by temperature shock that results when cultures are transferred from room temperature to 38°C and partly by the slight adjustments in cellular osmotic pressure which occur immediately following the change from *in vivo* to *in vitro* conditions.

In Fig. 3 is shown the relation between amount of yolk in the culture preparation to the mean number of cells completing mitosis in 352 minutes. It can be seen that the frequency of cells going through mitosis increased as the quantity of yolk increased up to ¼‡. The mean mitotic frequency in cultures containing ½ yolk was a little lower than that in the ¼-yolk cultures. This is probably caused by adverse effects of the slightly higher osmotic pressure that develops in the medium of the ½-yolk cultures about 3.5 hours after they are prepared. The cause of this increased osmotic pressure has not yet been determined. In cultures made up with amounts of yolk greater than ½, the increased osmotic pressure becomes manifest very soon after their preparation. For this reason larger amounts of yolk were not used in the present study.

Results of the experiments designed to de-

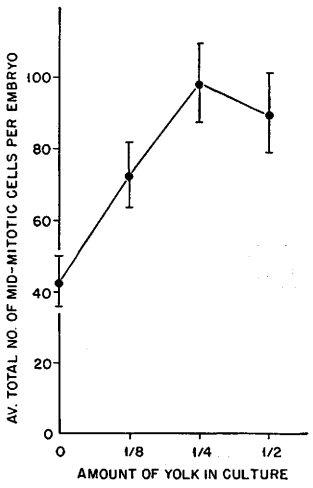

FIG. 3. Total number of mid-mitotic neuroblasts per embryo as function of amount of yolk in culture drop. The interval about each point represents the region within the 95% confidence limits.

‡ It should be noted that an amount of yolk approximately equal to ¼ that in an egg has been routinely used in preparing hanging-drop cultures in all previous work on grasshopper neuroblasts.

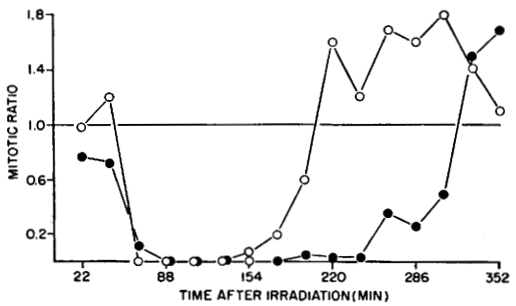

FIG. 4. Effects of yolk on recovery of neuroblasts from X-ray (64 r) induced injury. ○, mitotic ratio of X-rayed cells : control cells mounted with ¼ yolk; ●, mitotic ratio of X-rayed cells : control cells mounted without yolk. Data obtained from 8 embryos for each experimental condition.

termine the influence of yolk on recovery of neuroblasts from X-ray-induced mitotic inhibition are given as mitotic ratios in Fig. 4. It can be readily seen that the duration of the inhibition period is shorter and the beginning and completion of recovery is considerably faster in the neuroblasts of embryos mounted in yolk than in those of embryos mounted without yolk. These differences are statistically significant at the 1% level as shown by an analysis of variance of the raw data. In other words, yolk greatly enhances the recovery of neuroblasts from X-ray-induced mitotic injury.

Discussion. Untreated neuroblast. The decrease in mitotic rate which is evident soon after neuroblasts are separated from yolk suggests that the neuroblast does not go through a given mitosis entirely on energy previously stored within the cell. The frequency of mid-mitotic cells in the no-yolk cultures reaches a statistically significant low level at the time (88-minute count) that the frequency in the yolk cultures is at its maximum (Fig. 2). This high frequency in yolk cultures is the result of recovery of middle and late prophase cells from a temporary inhibition induced by change from *in vivo* to *in vitro* conditions. It would appear, therefore, that the absence of yolk considerably affects the ability of middle and late prophase cells to complete mitosis.

Under optimum *in vitro* growth conditions, cells which were in early prophase and earlier stages or even in the previous mitosis would be expected to reach mid-mitosis at the 88-minute and successive counts.§ Since the frequencies of mid-mitotic cells in the no-yolk cultures are very low at these counts, it appears that cells in early prophase and earlier stages are unable to complete mitosis in the absence of yolk.

Cells in stages from very late prophase through anaphase either require very little energy for their processes or they operate on energy stored within the cell during earlier phases. The second explanation seems the more likely, especially in view of the considerable amount of activity, such as chromosome movement and cleavage furrow formation, that occurs during these stages.

Several investigators are of the opinion that a cell is capable of completing mitosis without acquiring additional energy after the process has begun. Bullough(4,5) has data on mitosis in mouse epidermis which he interprets as evidence that these cells need considerable amounts of energy to initiate mitosis but very little energy to complete it. Swann(6,7) has proposed, on basis of his work with developing sea urchin eggs and that of Jacoby, *et al.* (8) on chick fibroblasts, that the energy necessary for a given mitosis is supplied by the "siphoning" of an energy "reservoir" which is stored during the previous mitosis. In other words, conditions which adversely affect the energy supply of the cell would not be detected until the subsequent division. The results herein reported suggest that in the grasshopper neuroblast at least the first half of prophase does not operate solely on energy stored within the cell but is dependent on an outside source of energy for continuance of mitosis.

That the energy for division is derived from glycolysis seems a reasonable conclusion to be drawn from the results of several workers (9-12). Tipton and St. Amand(13) have shown, however, that glucose does not influence respiration of the 14-day-old embryo of the grasshopper *Chortophaga viridifasciata.* This is not surprising in view of the findings

§ These calculations are based on durations of the various mitotic phases as given by Carlson and Hollaender, *J. Cell. and Comp. Physiol.,* 1948, v31, 149.

of Bodine(14) and Hill(15) that fat seems to be the main source of energy for grasshopper embryos of this age.

Fischer(16) has found that the growth of chick myeloblasts and osteoblasts *in vitro* is dependent on several amino acids, cysteine being the most essential. Shaw(17) has identified 17 free amino acids in the 14-day-old egg of *Chortophaga*. However, since his analysis is based on entire contents of the egg, it is difficult to estimate the possible role of these amino acids in mitosis of the neuroblast.

Preliminary experiments in this laboratory indicate that a filtrate‖ of fresh grasshopper egg contents gives the same result as ¼ yolk. The filtrate contains miscroscopic globules of yellow "yolk" material but no other visible components.

Irradiated neuroblast. It is not known whether recovery from radiation-induced mitotic inhibition involves the replacement of some vital substance which was destroyed by radiation or the elimination of some substance which caused the inhibition(18). The pronounced positive influence of yolk on recovery of neuroblasts from radiation damage demonstrates that in this cell some extracellular metabolite or metabolites greatly enhance repair of mitotic damage. This result strikingly parallels that of Stapleton and coworkers(19,20,21) who have found that the addition of beef or yeast extracts, or amino acids to culture medium following irradiation causes considerable increases in the number of bacteria which survive the radiation. The amino acids in the grasshopper egg(17) may, therefore, be important factors in the recovery of the neuroblast from radiation damage.

The shapes of the curves in Fig. 4 suggest that the response of neuroblasts to 64 r of X rays in the absence of yolk differs from the response in presence of yolk only with respect to the duration of the inhibition period. The temporary retardation of cells in very early, early, middle and late prophase is mainly responsible for the inhibition period in the presence of yolk. Whether the absence of yolk affects recovery of cells in all these stages to the same extent or whether it differentially affects recovery of certain ones cannot be determined from the present data.

Summary. 1. The frequency of mid-mitotic neuroblasts in grasshopper embryos in hanging-drop cultures increases as the quantity of yolk in the culture is increased up to a quantity equivalent to ¼ that in an egg. The influence of yolk on mitosis in the untreated neuroblast is interpreted as indicating that, in this cell at least, the first half of prophase does not operate solely on energy stored within the cell but is dependent on an outside source of energy for continuance of mitosis. 2. The pronounced positive effect of yolk on recovery of neuroblasts from radiation damage demonstrates that, in this cell, some extracellular metabolite or metabolites greatly enhance repair of mitotic damage.

‖ The extraembryonic contents of 14-day-old grasshopper eggs were mixed in 10 volumes of culture medium and passed through a fine Seitz filter. Culture medium rather than plain water was used because yolk and, in particular, serosal fluid contain much protein, presumably globulin, which readily coagulates in water.

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Metabolism of Trichloroethylene in the Bovine.* (22019)

T. A. SETO AND M. O. SCHULTZE.
Department of Agricultural Biochemistry, Institute of Agriculture, University of Minnesota, St. Paul.

It has been suggested(1) that the aplastic anemia which is observed in the bovine(2) fed certain specimens of trichloroethylene-extracted soybean oil meal is induced by residual trichloroethylene in the toxic meal. Prolonged feeding of relatively large amounts of trichloroethylene however failed to produce harmful effects in calves(3). This suggests that the bovine, like some other species which are relatively resistant to the toxic effects of trichloroethylene-extracted soybean oil meal (4) can metabolize trichloroethylene. In fact, urochloralic acid has recently been isolated from the urine of calves fed trichloroethylene and identified through degradation and synthesis of a derivative as 2′, 2′, 2′-trichloroethyl-β-D-glucopyranosiduronic acid(5). It was thus established that the bovine, like the dog(6), can metabolize trichloroethylene to trichloroethanol and excrete the latter as a conjugate. It remained to be determined however, to what extent this occurs and if trichloroacetic acid, a major metabolite of trichloroethylene in mice(7), rats(8), dogs (9) and man(10) is also produced by the bovine.

Methods. Four female Holstein calves weighing from 100 to 112 lb were placed into individual metabolism cages, fed 5 lb of whole cows' milk twice daily and given water *ad libitum.* Trichloroethylene was administered orally in gelatin capsules twice daily with the milk in the following total daily amounts: Calf A, 3 g for 5 days; calf B, 12 g for 5 days; calves C and D, 12 g for 4 days. The urine was collected in brown bottles under toluene. Separate 24-hour specimens were obtained before, during and after the period of administration of trichloroethylene. Aliquots of the urine were used for determination of trichloroacetic acid, "total trichloro compounds" and trichloroethylene; the latter was also determined in the toluene layer. The analytical methods used have been described recently(11). Briefly they consist in the quantitative application of the Fujiwara alkali-pyridine reaction. In our modification trichloroacetic acid, trichloroacetaldehyde, chloroform and trichloroethanol do not interfere with the determination of trichloroethylene. The latter as well as chloroform can be removed from the urine prior to the determination of trichloroacetic acid or of "total trichloro compounds." Trichloroethanol and urochloralic acid do not interfere with the determination of trichloroacetic acid but all 3 compounds are included in the determination of "total trichloro compounds." The difference between "total trichloro compounds" and trichloroacetic acid (calculated on a molar basis as trichloroethanol) corresponds to free and combined "trichloroethanol" including urochloralic acid. Other products of metabolism of trichloroethylene which no doubt exist have not been definitely recognized. If, after oxidation they would give a positive Fujiwara reaction they would be included as "total trichloro compounds" and would thus contribute

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