INDUCTION OF DOMINANT LETHAL MUTATIONS IN INSECT OOCYTES AND SPERM BY GAMMA RAYS AND AN ALKYLATING AGENT: DOSE-RESPONSE AND JOINT ACTION STUDIES

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IT is generally recognized that certain types of chemicals cause biological effects closely resembling those produced by radiation. Among these effects are the production of mutations, chromosome aberrations, and induction of sterility and cancer in animals. Our interest has been mainly to determine the effect of gamma radiation and alkylating agents upon growth and maturation of insect reproductive cells and the production of sterility by inducing dominant lethal mutations in these cells. In undertaking the studies reported herein we had several immediate objectives: (1) To determine dose-response curves for induction of dominant lethal mutations by either radiation or tretamine (2,4,6-tris(1-aziridinyl)-*s*-triazine) in meiotic oocytes and mature sperm for comparison of slopes, reaction kinetics and median lethal doses; (2) to compare response of different cell stages to either mutagen; and (3) to derive preliminary information from dose-response curves for later tests to determine potentiation of one agent by the other. No published study has come to our attention in which an inquiry was made into the combined action of a chemical mutagen and radiation in the induction of dominant lethal mutations.

MATERIALS AND METHODS

Adult male and female screw-worm flies, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), were used in these experiments. The female reproductive system contains two ovaries, each consisting of 100 to 150 ovarioles in which egg development occurs synchronously. By treating one female, hundreds of oocytes, all in the same stage of development, are simultaneously exposed to the treatment. Gravid females deposit 200 to 250 eggs in a single mass.

Three-day-old females reared at 80°F contain oocytes with the nucleus in early prophase of the first meiotic division, 4-day-old females contain almost fully mature eggs with the nucleus in metaphase I, and 5-day-old females contain a large number of fully mature eggs in which meiosis has progressed up to early anaphase I (LACHANCE and LEVERICH 1962). Further growth and changes in the egg and its nucleus are arrested in this stage until the egg is laid. Thus, the induction of dominant lethal mutations in meiotic oocytes can be detected by treating virgin females, mating them to untreated males, and scoring the hatchability of the eggs produced. Similarly, dominant lethal mutations in mature sperm are detected by treating 1-day-old males and allowing a single mating between treated males and normal females.

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In the present studies, flies were allowed to emerge from puparia for 4 hours, sexed the same day, and caged with undiluted honey and water in a colony room at 80°F. Considerable care was taken to insure that all flies in a group were of the same age ± 2 hours, kept under uncrowded, well-fed conditions to insure maximum synchrony in physiological age and development. Males were treated at 24 hours of age and females at exactly 3 or 5 days of age to study the effect on mature sperm, early prophase oocytes, and early anaphase oocytes, respectively. The three cell stages were known to differ considerably in their sensitivity to mutagens, but it was not known whether the dose-response relationship with a chemical mutagen was similar to that of radiation. Approximately 40 to 50 flies were treated with each dose in each experiment.

For radiation treatments, flies were transferred to cylindrical screened containers and exposed to Co^{60} gamma radiation in air. Since our studies extended over an 8-month period, during which available radiation sources were being recharged, several different sources were used. Dose rates ranged from 457 to 1,158r per minute. A control group consisting of untreated males and females was run concurrently with each radiation experiment.

For tretamine treatments, flies were anesthetized by chilling and 2.0 μl of freshly prepared methanol solution was applied to the dorsal thorax of each fly. Dosage was controlled by changing the concentration of the chemical. Control flies for the chemical series were treated with 2.0 μl of methanol alone. Further details of chemical treatment are given elsewhere (LaChance and Crystal 1963).

When flies received both treatments, the chemical was applied first; flies were then transferred to containers for irradiation and transported to the radiation source for treatment. A uniform interval of 16 minutes was allotted between the end of the chemical treatment and the beginning of the radiation treatment.

After treatment, flies were placed in cages with water and honey and held in a colony room at 80°F until they were 5 or 6 days old. Sixteen to 18 hours before collection of eggs, an equal number of untreated flies of the same age but of the opposite sex were introduced into the cages with the treated flies. This procedure reduced the chances of multiple matings by males, since females are monogamous, and thus assured the transfer to females of sperm which were mature at the time of treatment. (Cytological studies conducted subsequent to the present experiments [Riemann unpublished] suggest that a delay between the treatment of males and their mating may possibly result in a mixing of immature sperm with the mature sperm sampled in the single mating by the adult male. However, the extent to which this occurs in this species and its effect on the present experiments is unknown.) In addition, this procedure reduced chances that the untreated group would become contaminated with the chemical by contacting treated individuals (Crystal 1964). When females were treated at 5 days of age, untreated males were added 30 hours later.

When females were 6 or 7 days old, 20 females from each group were induced to oviposit by placing each female in a vial with a small amount of ground lean meat and holding the vials at 90° to 96°F for a few hours. After the female produced an egg mass, she was killed and the spermathecae dissected and examined for the presence of sperm. Only egg masses from inseminated females were used to determine hatchability. This procedure was adopted to insure a sampling of fertilized eggs. Experience has shown that fertilization of eggs by sperm from treated males is not adversely affected at the doses used in these experiments. Egg masses were pooled and the eggs were separated by soaking in 1% aqueous sodium hydroxide solution and then rinsed several times with distilled water. From this thoroughly mixed sample of about 4,000 eggs, a random sample was removed with a dropping pipette and the eggs aligned in rows on a damp black cloth in a petri dish. About 200 eggs were placed in each of five dishes. Hatchability of eggs was determined after incubation for 24 to 28 hours at 80°F.

For each experiment an estimate of dominant lethality induced by a given dose was based on the average hatch of about 1,000 randomly selected eggs subdivided into five groups. Each estimate was then corrected for egg hatch noted in the parallel control. Results of several replicate experiments (2 to 7 replicates for each dose) were pooled and a grand mean with standard error (shown as vertical lines in figures) was computed. (For economy of space the lengthy tables

presenting the raw data for each replicate have been omitted from the presentation. The tabulated data are available on request from the authors). In all, 23 experiments were completed, at the rate of about one per week, between December 14, 1962 and July 26, 1963.

RESULTS

Dose-action studies: Results relating dose to percentage of dominant lethals induced in different germ cell stages are presented in Figures 1 to 3. Examination of these figures reveals one general feature. The amounts of genetic damage induced by low doses of radiation can be replicated with greater accuracy than those by low doses of tretamine. Only at higher doses of tretamine can one expect more consistent results. The variability may be due in part to variations in cuticular penetration, detoxification mechanisms, and cellular permeability factors inherent in topical treatments with chemicals. In his work with Drosophila, OSTER (1958) found that "with chemical treatments duplication of dosage from experiment to experiment is fraught with difficulty and uncertainty." The same difficulties are not encountered in radiation experiments.

In plotting data for Figures 1 to 3, it was obvious that the shape of the curve could be considerably altered by adjusting the dose scale. Doses of tretamine have not been equated with doses of gamma radiation; the graphical presentation simply relates increase in genetic damage as a function of dose.

Data presented in Figure 1 permit a comparison of the doses of radiation or tretamine required to induce a given percentage of dominant lethal mutations in sperm. Following irradiation, genetic damage increases rapidly as a function of the dose up to 3,000r. Beyond 3,000r the curve levels off markedly, and further increases in dose do not result in substantial increases in lethal mutations. From 1 to 2 kr the percentage of lethals increased as the 0.90 power of the dose, whereas when the dose increased from 2 to 4kr or 3.5–7kr the frequency of lethals increased only as the 0.69 and 0.57 power of the dose. (The power of the dose exponents were calculated by the method of MULLER [1940]).

Low concentrations of tretamine (0.00625 to 0.0125%) induced low rates of lethals. As the dosage of tretamine was increased above 0.02%, results were fairly consistent and the percentage of dominant lethals induced in sperm increased rapidly until a dosage of 0.05% was reached. At still higher doses the curve levels off considerably, suggesting a saturation effect. As with radiation, as the dose at lower concentrations (0.006 to 0.05%) is doubled the percentage of lethals increases as the first power of the dose, whereas doubling the dose in the range of 0.05 to 0.15% resulted in an increase of 0.5 power of the dose. With tretamine the 99% lethal dose for mature sperm was 0.3% and with radiation, 7kr. The dose-action curves for sperm treated with either gamma radiation or tretamine are nonlinear and quite similar in shape.

Data presented in Figure 2 describe the response of immature oocytes (early prophase stage) to induction of dominant lethals by gamma rays and tretamine. Again the curves are remarkably similar but assume a different, sigmoidal shape. Response of immature oocytes in 3-day-old females was characterized by marked resistance to effects of both mutagens. At low doses (2 to 4kr or 0.003 to 0.04%

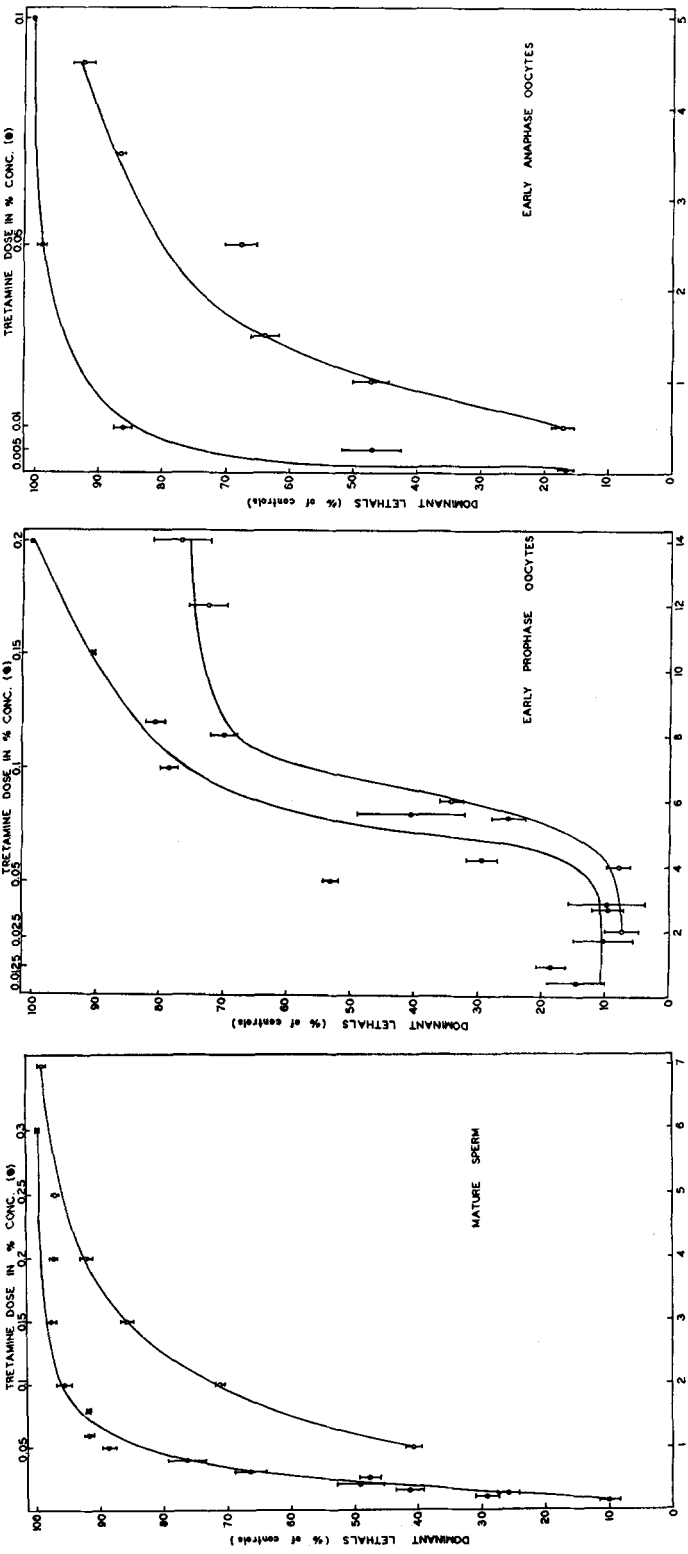

FIGURE 1.—Induction of dominant lethal mutations in mature sperm by gamma radiation and tretamine (2.0 μl applied topically). One-day-old males treated. (○ = gamma rays; ● = tretamine).

FIGURE 2.—Induction of dominant lethal mutations in immature oocytes (early prophase stage) by gamma radiation and tretamine (2.0 μl applied topically). Females treated when 3 days old. (○ = gamma rays; ● = tretamine).

FIGURE 3.—Induction of dominant lethal mutations in mature oocytes (early anaphase stage) by gamma radiation and tretamine (2.0 μl applied topically). Females treated at 5 days of age. (○ = gamma rays; ● = tretamine.

tretamine) the percentages of dominant lethals did not increase greatly. However, almost nine times as many lethal mutations were induced at 8kr as at 4kr, and in this dose range the frequency of dominant lethals increases as the square of the dose.

With tretamine, at concentrations from 0.025 to 0.08%, the percentage of lethals increased as the 1.7 or 1.5 power of the dose. The increase was much less (0.67 power of the dose) at higher doses. A dose of 0.2% of tretamine induced 100% dominant lethals. The slope of the dose-response curve for gamma radiation levels off sharply at doses above 8kr, whereas that for the tretamine maintains a fairly constant slope at high doses. Possibly the γ-radiation curve for early prophase oocytes would maintain a more constant slope at higher doses if the treatment were administered at a higher intensity.

Data on induction of dominant lethal mutations in mature screw-worm oocytes (early anaphase I stage) by gamma radiation and tretamine are presented in Figure 3. This cell stage is characterized by extreme sensitivity to gamma radiation and tretamine, as well as other chemical mutagens (LaChance and Crystal 1963). At very low doses of tretamine (below 0.0005%), results were highly variable. This variability was due to extreme sensitivity of cells, perhaps compounded by possible errors in dilution, application, or penetration of the chemical. Therefore, in plotting the data for Figure 3 we omitted results with the lower concentrations of tretamine because of the inordinate amount of variability in this sensitive stage. At higher doses of tretamine (0.001 to 0.01%) the percentage of genetic damage increased very rapidly (power of the dose exponent for 0.005 to 0.01% is 0.93). At doses of tretamine above 0.01%, the curve levels off sharply since damage increased only very slightly as the dose was doubled.

After irradiation of early anaphase I oocytes, genetic damage increased very rapidly in the range from 500 to 1,000r; but then the rate of increase was reduced as the dose increased. (The power of the dose factors for the 0.5 to 1, 1 to 2 and 2 to 4kr are 1.2, 0.8 and 0.67, respectively.) The dose-action curves for both tretamine and gamma radiation are nonlinear. The tretamine curve has a phenomenally steep slope that levels off markedly at the highest doses. The gamma radiation curve is characterized by a less steep slope that does not level off as abruptly at the higher doses.

The data strikingly point out the great differences in susceptibility to mutagens among the various types of reproductive cells. Immature oocytes in early prophase I stage were more resistant than mature oocytes and sperm. Mature sperm were about six times more sensitive to radiation treatments than oocytes in prophase I. Oocytes in anaphase I were about five to seven times more radiosensitive than those in prophase I. Anaphase I oocytes and mature sperm were very nearly equally sensitive to ionizing radiation. After treatments with tretamine, mature sperm were about two to four times more sensitive than oocytes in prophase I. Oocytes in anaphase I were consistently (9 to 25 ×) more sensitive than those in prophase I.

Tests for joint action of gamma radiation and tretamine: Doses of gamma radiation and tretamine that would induce less than 50% dominant lethal muta-

tions were selected on the basis of the dose-response curves. Flies were treated with half doses, single doses, or a combination of a half dose of each agent. Mature 5-day-old females were not included in these tests since the concentration of tretamine required to induce 50% lethals is extremely low and thus the variability of results so great as to be unreliable for comparisons.

Tests with mature sperm were replicated four times and those with immature oocytes three times. However, in some of these trials, tretamine treatments induced very low frequencies of lethal mutations indicating loss of potency of the

TABLE 1

*Tests for joint action of gamma radiation and tretamine on mature sperm**

Dose	Dominant lethals (% of controls)	
	Trial 1	Trial 2
Radiation:		
1,000r	36.8	51.4
2,000r	69.0	74.9
Tretamine:		
0.015 percent	41.8	...
0.03 percent	59.7	72.6
0.06 percent	...	89.7
0.015 percent tretamine + 1,000r:		
Observed	63.6	...
Expected	64.4	...
0.03 percent tretamine + 1,000r:		
Observed	...	80.2
Expected	...	82.3

* One-day-old males each treated with 2.0 μl and later mated to untreated females.

TABLE 2

*Tests for joint action of gamma radiation and tretamine on immature oocytes**

Dose	Dominant lethals (% of controls)	
	Trial 1	Trial 2
6,000r	41.6	23.4
12,000r	73.3	84.7
0.06 percent tretamine	35.0	23.3
0.12 percent tretamine	67.9	82.2
0.06 percent tretamine + 6,000r:		
Observed	55.1	65.7
Expected	70.6	83.5

* Three-day-old females each treated with 2.0 μl and later mated to untreated males.

chemical in some batches used. Results of the four trials in which the chemical was effective are presented in Tables 1 and 2.

Existence of potentiation was tested by the summation method (MITCHELL and SIMON-REUSS 1952), a recognized technique of pharmacology and radiobiology. The method entails comparing the total effect of half doses of the two agents with the mean of the single doses of each agent, giving the expected values in Tables 1 and 2. Results indicated equal or less damage after a combined treatment of gamma radiation and tretamine than would be expected on the basis of additivity of the two mutagens. Both experiments with mature sperm resulted in the two agents having an additive effect. The two experiments with immature oocytes (Table 2) results in the two agents having a less than an additive effect.

DISCUSSION

Dose-action studies: When dominant lethal mutations are induced in mature sperm and meiotic oocytes by either gamma rays or tretamine, the shapes of the two dose-response curves are very similar for a given cell stage, but different cell stages exhibit different shapes and different levels of sensitivity to these mutagens. Data presented in Figures 1 to 3 indicate that dominant lethal mutations did not increase linearly with the dose, at least not over the entire range of doses. The dose-action curves for mature sperm and anaphase oocytes approach linearity only at the lower doses tested, but the curves for these cells over the entire range of treatments may be more accurately described as exponential.

SONNENBLICK (1940) found that the dose-action curves for X-irradiated mature sperm and oocytes of *Drosophila melanogaster* were nonlinear. Induction of dominant lethals in mature sperm, after injection of tretamine into the abdomens of Drosophila males, followed nonlinear curves very similar to those of X-irradiated males (FAHMY and FAHMY 1954). They interpreted these dominant lethal curves as follows: "This suggests that dominant lethals, whether induced by the physical or the chemical agent, are predominantly single-event effects at low doses, as would result from single-break chromosome aberrations, but at higher doses are the consequence of multiple effects, as would result from multiple breaks and exchanges."

Our results (see Figures 1 and 3) indicate that single-break events are sufficient to induce dominant lethal mutations in mature sperm and anaphase I oocytes of screw-worm flies. The saturation of these curves at high doses may be due to multiple-break events, or the induction of several single-hit events in the same cell which are superfluous.

The shape of the curve obtained after treatment of prophase I oocytes with gamma radiation or tretamine is distinctly different than the curves obtained with mature sperm and anaphase I oocytes. The curve (see Figure 2) shows a clear threshold at lower doses. The response of prophase I oocytes at low doses suggests a two-hit basis for dominant lethals. In their investigations on prophase I oocytes in Habrobracon and stage-7 oocytes in Drosophila, WHITING (1945) and PARKER (1959) presented data which yield radiation dose-action curves very

similar to the one in Figure 2. Thus, for at least three insect species in which clearly defined cell stages can be irradiated the shape of the dose-action curve is similar although the doses required to produce a given level of lethal mutations vary considerably between the species.

In meiotic oocytes and sperm of *Cochliomyia hominivorax*, LaChance and Riemann (1964) found that dominant lethal mutations were characterized by the presence of chromosome bridges and fragments between dividing nuclei in the embryo. However, the stage of development at which the aberrations first appeared depended on the type of mutagen used. Chromosome damage produced by γ-irradiation of meiotic oocytes (in 3- or 5-day-old females) became evident during the first and second meiotic divisions (completed after oviposition) and continued into early cleavage divisions, at which time development ceased. After treatment of meiotic oocytes with tretamine, most meiotic divisions appeared normal, and chromosome aberrations were not usually found until the first cleavage divisions, or after the treated nucleus had undergone one replication of chromatin material. Damage to chromosomes of mature sperm with either gamma rays or tretamine becomes evident at the first cleavage divisions, the earliest stage at which damage induced in mature sperm can be observed cytologically. Therefore, chromosome breakage is the basis of dominant lethality in different kinds of reproductive cells; but expression of chromosome breaks induced by radiation are "immediate" in nature whereas those induced by tretamine are commonly "delayed."

Although the mode of action of the two mutagens is apparently different, the pronounced similarity in the dose-response curves is perhaps a reflection of the end effects measured.

Similarity of dose-action curves may indicate that the mutagens have the same site of action (chromosomes), although the time at which the chromosome aberrations appear after treatment of the cell is different. One consistent difference observed was that the radiation curves saturate earlier (at a lower level of damage) than do the tretamine curves.

Interaction of tretamine and gamma rays: Absence of potentiation between tretamine and radiation applied consecutively may be attributed to a difference in their mode of action or in the time at which genetic damage becomes evident. LaChance and Riemann (1964) found that although tretamine induced chromosome breaks in the fully formed chromosomes in eggs and sperm, it more generally induced a "delayed" type of chromosome breakage, not expressed until cleavage divisions in the embryo. Evans and Scott (1964) found that maleic hydrazide also produces chromosome aberrations at the time of chromosome replication—not at the time of treatment. An alternative explanation is that chromosome damage induced by tretamine was usually of the chromatid or subchromatid type, the expression of which would also be delayed. In either case, it is more reasonable to expect additivity of effects.

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SUMMARY

Adult female screw-worm flies containing oocytes in early prophase or early anaphase of the first meiotic division, and 1-day-old males containing mature sperm were exposed to graded doses of gamma radiation or the alkylating agent, 2,4,6-tris (1-aziridinyl)-*s*-triazine (=tretamine). Frequency of dominant lethal mutations induced by these treatments was determined by scoring hatchability of eggs produced by inseminated females. Crosses involved treated females × untreated males or the reverse. Dose-response curves permit a comparison of radiation and tretamine treatments on the three stages of reproductive cells that differ greatly in their sensitivity to both radiation and chemical mutagens. Shapes of curves for both radiation and tretamine treatments are remarkably similar for the same cell stage. The relationship between dose and percentage of dominant lethals was nonlinear for all cell stages.

In testing for possible potentiation of one treatment by the other when treatments were applied consecutively, adult males and females were exposed to tretamine followed in 16 minutes by an exposure to gamma radiation. Doses used were known to produce less than 50% dominant lethals when given alone. Treated insects were crossed to untreated ones and frequency of dominant lethal mutations induced by combined treatments compared with those by single treatments was assessed. Results indicated that the effect of combined treatments on oocytes and sperm was merely additive, at best.

Possible reasons for similarity of the dose-response curves elicited by basically different types of mutagens, shapes of curves, and absence of potentiation when treatments included both radiation and tretamine are discussed.

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