DIFFERENTIAL SENSITIVITY OF MOUSE SPERMATOGENESIS TO ALKYLATING AGENTS

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VARIOUS alkylating agents have long been used for inducing mutations and chromosome breaks in some plants and animals. Effects on Drosophila spermatogenesis have been investigated by AUERBACH (1940, 1949, 1958), especially with mustard gas, and FAHMY and FAHMY (1953, 1956) used various compounds. All types of mutations have so far been obtained—dominant lethals and semilethals, recessive lethals, visible viable mutations, and chromosome breaks.

Such intensive investigations have not been achieved in the mouse, in which the study of mutagenesis with radiomimetic compounds is just commencing. BATEMAN (1958) recorded dominant lethals and found some analogy in the distribution of breaks per egg between X rays and a polyfunctional imine, Triethylenemelamine (TEM). CATTANACH and EDWARDS (1958) found that TEM reduces the fertility of the male mouse.

The present work deals with a preliminary study of chromosome breaks observable at various times after treatment of spermatogenic cells of the mouse with alkylating agents. Some chemical tests *in vitro* and some experiments in plants suggested that polyfunctional compounds are more active than monofunctional ones. However, this idea is not universally accepted. For this experiment, in spite of this possible discrepancy, three polyfunctional compounds belonging to three quite different chemical classes were arbitrarily selected. Comparison with monofunctional chemicals will be reserved for future investigations.

MATERIALS AND METHODS

Myleran (Busulfan) (1,4-dimethanesulfonoxybutane): This substance has been used frequently in hematology as an antileukemic agent. Mutagenic activity of alkylmethanesulfonates was tested in Drosophila by FAHMY and FAHMY (1956). Some special properties of Myleran were reported by MOUTSCHEN and MOUTSCHEN-DAHMEN (1958).

Triethylenemelamine (2,4,6-triethyleneimino-1,3,5-triazine—TEM): Mutagenic and carcinogenic activity of this compound was tested by FAHMY and FAHMY (1954, 1955a,b). It has also been used in plant experiments.

Diepoxybutane (1,2,3,4-butadiene dioxide): This drug was tested in the plant

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kingdom, where REVELL (1953) carried out a cytological analysis of the induced lesions in *Vicia faba.* FAHMY and FAHMY (1953) also tested it for mutagenic and carcinogenic activity in Drosophila. Diepoxybutane also has been used for induction of gene mutations in Neurospora by KØLMARK (1953); KØLMARK and GILES (1953); KØLMARK and WESTERGAARD (1953); WESTERGAARD (1957).

Hybrid 101 × C3H mice were injected intraperitoneally when 5 to 7 months old and then killed at different times after treatment. After immersion for ½ hour in ½ isotonic NaCl solution, testes were fixed in acetic acid-alcohol (1:3) for 1 to 2 hours. They were then stained *in toto* with Schiff-Feulgen and observed after squashing.

The use of anaphase fragments and bridges for estimating damage caused by alkylating agents probably is not the best in absolute values, but it avoids some possibilities of mistakes that exist when metaphase lesions are considered. For instance, in metaphase the early separation of the heterochromosomes makes it difficult to distinguish these structures from fragments.

RESULTS

A. *Sensitivity of different stages of spermatogenesis:* In a first set of experiments, the following doses of chemical were used: diepoxybutane, 5 mg/kg; TEM, 6 mg/kg; Myleran, 16 mg/kg. Animals were killed at 20 hours and 2, 5, 10, and 20 days after injection. A few animals were killed at 3, 4, 7, and 30 days. Chromosome breaks estimated by anaphase fragments are plotted in Figure 1A, which shows that (1) the sensitivity of various stages is different for the three compounds; and (2) for Myleran, there are two peaks of sensitivity. The sensitivity diminishes from a stage presumably close to the resting stage of spermatocyte I to the middle of pachytene and then increases to another maximum. The peak at 1 to 2 days, according to OAKBERG's estimate (1956) of duration of spermatogenesis, must be near the end of pachytene or the beginning of diplotene. It has not been possible to determine the sensitivity of spermatogonial cells to chromosome breakage since these cells are killed by TEM and Myleran. Moreover, in such an attempt, we should have to compare the sensitivity of mitotic and

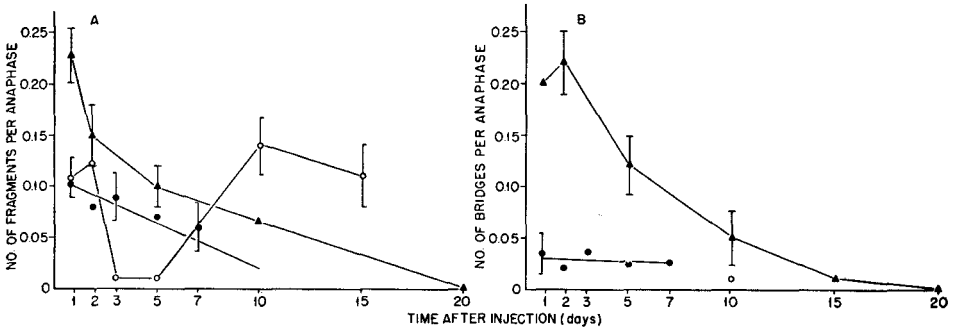

FIGURE 1.—Frequency of fragments and number of bridges in anaphase I after different times with diepoxybutane (▲), TEM (●), and Myleran (○).

meiotic divisions, which are not strictly comparable. Macroscopically observed, testicular atrophy occurs about 15 days after Myleran injection. (3) For TEM, there was testicular degeneration at 10 to 15 days. No sensitivity of early spermatogenesis to this chemical could be ascribed. (4) For diepoxybutane, no testicular degeneration was observed after 20 or 30 days or even longer times up to 50 days. For this compound, only one peak was found, which must be situated around diplotene; it corresponds to the peak for Myleran at day 1, but differs from Myleran in that a significant decrease in fragments but an increase in bridges occurs by day 2.

Although some fragments were scored in spermatogonial anaphases, the intensity of this damage was not severe enough to induce testicular degeneration. It is possible that DNA synthesis occurs at the time when a second peak has been found for Myleran, i.e., before leptotene, but it is unlikely that DNA synthesis is involved in the day-1 peak. Thus no conspicuous relation between peaks of sensitivity and the presumed time of DNA synthesis could be found. The difficulty of a possible disturbance of duration of spermatogenetic period by the poisons could not be avoided. These disturbances, which do not occur or at most are slight with X rays (EDWARDS and SIRLIN 1958; OAKBERG and DIMINNO 1960), would necessarily lead to difficulties in identification of cell stages at time of treatment. If they occur, the exact intensity of this kind of inhibiting effect on meiotic processes would have to be known. Presumably, the amount of inhibition could be different not only from one compound to another but also from one dose to another. In this first set of experiments, however, the injected amounts of drugs were not very toxic for the animal, especially Myleran, and we therefore think that the conclusions have not been seriously affected. A somewhat similar experiment was carried out with X rays in order to obtain a reference scale for estimating the intensity of damages induced with toxic substances.

B. *Relative efficiencies of chemicals and X rays:* In this set of experiments, as in the previous one, the average number of anaphase fragments was taken as the criterion for estimating the damage. These cells were scored 20 hours after injection since, as shown in Section A, at this time a peak of highest sensitivity for the three chemicals was obtained. The data are pooled in Figure 2A and show the results. (1) For the three chemicals, the fragment frequency increased with increasing dose. According to the restrictions made in Section A, after 20 hours the intensity of a possible meiotic disturbance cannot be very high. (2) The increase with dose is higher for diepoxybutane than for TEM and higher for TEM than for Myleran. (3) It seems to be difficult to exceed a certain level of effect. For diepoxybutane, a plateau was rapidly reached and was not exceeded, owing to a strong killing effect on the animals. Myleran doses were limited in these experiments by the low solubility of the substance. (4) At higher doses, TEM gave more lesions than the other compounds, but at these doses its toxicity for the animals is so high that it eliminates all practical possibilities of mutation induction.

These experiments show that, in future investigations on induction of mutations in the mouse with radiomimetic compounds, we will always be subject to

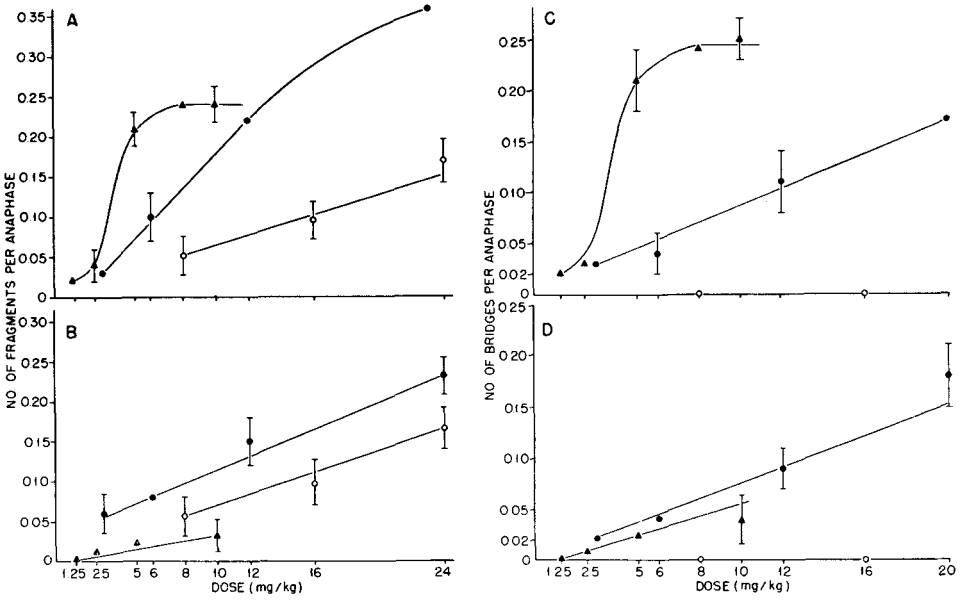

FIGURE 2.—Frequency of fragments in anaphase I (A, C, 300 cells) and anaphase II (B, ~600 cells; D, 300 cells) after varying doses of diepoxybutane (▲), TEM (●), and Myleran (O).

limiting factors, some of which are already well known from other work with plants and animals. We may be favored, however, by some special circumstances that result from the sensitivity of mammalian cells to specific compounds. If the activities of the chemicals are compared at the highest possible dose (say 10 mg/kg), diepoxybutane is the most efficient compound for breaking chromosomes, being about 1.5 times as efficient as TEM and four times as efficient as Myleran. Below these limits, the lower the dose, the more reduced are the respective efficiencies.

The following observations result from a comparison of the efficiency of the three compounds with that of X rays. (1) The efficiencies of the compounds on the second interphase were comparable with those observed for the first, but TEM was a little more efficient than diepoxybutane (Figure 3). The second meiotic division was chosen for this set of experiments because it is shorter. (2) The second meiotic cycle (including also probably late spermatocyte I stages) can be considered somewhat more sensitive to the drugs than the first one. This situation is similar to that obtained for ionizing radiations (OAKBERG and DIMINNO 1960).

The results summarized in Figure 3 show that the presumed sensitivity of the second interphase to toxic chemicals is not very great as compared with the sensitivity to radiations. This comparison is limited, however, to doses of chemicals low enough not to be toxic to the animals. This comparison does not afford all possibilities and therefore has no absolute value. Only the immediate numbers of lesions scored in anaphase II were compared. In fact, it is known that, with toxic substances, some delayed effects can exist that this scoring cannot take into

account. This method, therefore, probably underestimates the real number of lesions.

C. *Elimination of damage:* Fragment elimination could be estimated by comparing the average number of fragments in anaphases I and II. Two sets of experiments were performed. The first one represents a comparison, 20 hours after injection, of the effects of increasing the dose. In the second, the effects of stabilizing the dose while increasing the intervals were studied. Since a delayed effect can exist for chemicals, however, the present data measure not only the elimination of damage but also a combined action of elimination and new genesis. Elimination includes selective processes, nonvisible fragments at the poles, and probably early cell degeneration. The new genesis includes delayed appearance of lesions not visible before and also presumably subchromatidic lesions. The elimination of fragments is quite different for the three chemicals. Figures 2A and B summarize the results: (1) Elimination is higher for diepoxybutane than for TEM or Myleran. (2) For Myleran, there are no significant differences between the counts in anaphases I and II. This means that, with this compound, new genesis can be higher or elimination lower than for the other compounds, or both. This result agrees fairly well with some previous data in barley and the broad bean (MOUTSCHEN and MOUTSCHEN-DAHMEN 1958). (3) For diepoxybutane and TEM, the higher the dose, the more efficient is the elimination of fragments.

D. *Rejoining ability of broken ends:* In the previous experiments, the number of anaphase fragments was taken as the criterion for estimating the damaging effect of radiomimetic compounds. In the course of these experiments, it was also

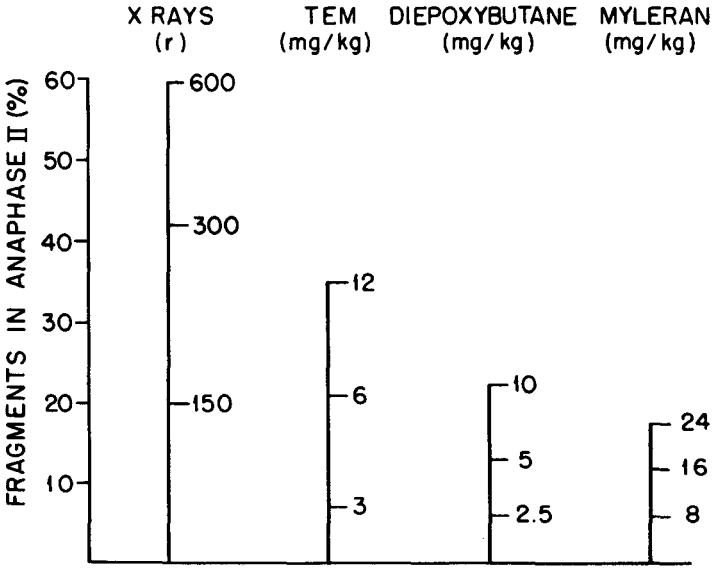

FIGURE 3.—Comparison of the relative efficiencies of X-rays and chemicals ten hours after treatment (300 anaphases).

possible to score the anaphase bridges and then, in some cases, to estimate the rejoining ability. The following findings came from the experiments.

(1) For diepoxybutane, if bridges are scored at different intervals after injection, as was done for fragments, the shapes of the curves are almost the same. (2) For TEM, there are comparatively fewer bridges. (3) For Myleran, the situation is obviously quite different. With this chemical, no bridges were observed until 10 days after injection. At all times, the number of observed bridges remains significantly lower than for the two other compounds; it may not be significantly higher than in the control. This peculiar situation is somewhat comparable to that described in barley and in the broad bean (MOUTSCHEN and MOUTSCHEN-DAHMEN 1958). (4) With both TEM and diepoxybutane, bridge frequency seems to parallel break frequency. The ratio of breaks to bridges is close to unity for diepoxybutane. Relatively, this substance seems to give the highest proportion of rejoinings. No bridges were observed with Myleran.

Figures 1B and 2C and D summarize the results. In Figure 1B, bridges were scored at several times after injection, as mentioned before. In Figures 2C and D, they were scored 20 hours after injection, respectively, in anaphases I and II. In anaphase II, instead of an increased number of bridges, a decrease can be noted. This suggests that, after one meiotic cycle, some broken bridges did not rejoin and could be due to some inherent properties of broken ends.

DISCUSSION

The stages yielding the most reliable data extend over the entire spermatocyte cycle, including maturation divisions. For both prespermatocyte and postspermatocyte stages, the sensitivity can be estimated only by some indirect means. For instance, a high sensitivity of spermatogonia to TEM and Myleran is indicated by testicular atrophy. In this respect, diepoxybutane acts quite differently. This could be a valuable property in future investigations, whereas the higher sensitivity of spermatogonial stages to TEM and Myleran could complicate future work with these substances. The efficacy of diepoxybutane in breaking chromosomes has been reported previously for other material. In Neurospora, mutagenic activity of the epoxides is very well known (JENSEN, KIRK, KØLMARK and WESTERGAARD 1951; KØLMARK and WESTERGAARD 1949; KØLMARK and GILES 1955).

With chemicals, a delayed effect in producing mutations and chromosome breaks has been definitely demonstrated. In plants, for instance, KIHLMAN (1952) found delayed breakage with a purine derivative, 8-ethoxycaffeine. With the same compound, it has been shown that some effects can be so delayed that they appear at the first generation after treatment (MOUTSCHEN and MOUTSCHEN-DAHMEN 1960). In Drosophila, treatment of spermatozoa with chemicals can show effects very late in ontogeny. Therefore, in these experimental results, a possible delayed effect must not be overlooked. It has been supposed that new genesis of breaks could occur in anaphase II as a result of subchromatidic breaks induced during the first spermatocytic stages. In this case, some attached frag-

ments should be seen at anaphase II. Since these fragments failed to appear, the occurrence of subchromatid breaks is very improbable. In plants, however, Myleran induced delayed chromosome breaks although few attached fragments were observed (MOUTSCHEN and MOUTSCHEN-DAHMEN 1958). Therefore, at least in Myleran experiments, other causes of delayed effects cannot be ruled out. This would explain the results of the comparisons between fragment numbers observed in anaphases I and II 20 hours after treatment.

At equal mortality, the efficiency of chemicals in production of chromosome breaks does not seem high compared with X rays. Here, too, a possible delayed effect of chemicals should not be disregarded for complete comparison. In plants, chromosome breaks induced with alkylating agents were not found to be randomly distributed but specifically localized at some "weak spots" (see FORD 1949, for mustard gas; REVELL 1953 for *NN*-di(2-chloroethyl)methylamine and some epoxides; OCKEY 1957, for some imino compounds; MOUTSCHEN and MOUTSCHEN-DAHMEN 1958, for Myleran). The same was found for Drosophila mutations by FAHMY and FAHMY (1957a), although the reliability of these last findings is not admitted by all investigators.

At the present time, there is no information on the qualitative aspects of chromosome breakage in the mouse. It would be worth extending these investigations to larger mammalian chromosomes.

Comparison of the sensitivity of various stages of spermatogenesis in the mouse with the organism in which it has been most investigated, i.e., Drosophila, could be valuable. In this material, AUERBACH (1958) showed that spermatozoa are very sensitive to mustard gas. A peak of higher sensitivity occurred in a stage before maturation of spermatozoa, probably spermatids. In Drosophila, too, the sensitivity of the same stages of spermatogenesis differs from one toxic compound to another. For instance, after 2-chloroethylmethanesulfonate, mutations were observed only in late broods or those arising from treated spermatogonia (FAHMY and FAHMY 1957b). The situation in the mouse might be comparable. It could be argued that, since chromosome breaks represent only a part of mutagenesis, these data have to be extended to all types of mutations. At the present, we could only speculate about the possible causes of differential sensitivity to the toxic compounds. It seems logical that a metabolically highly active tissue would be most sensitive. This explanation does not seem very plausible, however, when applied to the end period of pachytene and diplotene.

SUMMARY

F_1 males obtained by the cross of mouse strains 101 and C3H were injected with Myleran, diepoxybutane, and Triethylenemelamine (TEM). Chromosome fragments and bridges were scored in anaphases I and II and taken as a measure of the intensity of damage induced by the chemicals. Examinations were carried out at progressive intervals after injection of single doses of the three compounds and after 20 hours for progressively increased doses of the compounds.

A differential sensitivity of spermatogenic stages and some differences in ac-

tivity occurred. According to these differential responses, some future applications in genetics can be suggested.

At the same time, an attempt was made to estimate the elimination of lesions by comparing the numbers of lesions at anaphases I and II 20 hours after injection.

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