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ABSTRACT

Experiments of Glass and Plaine (1950) yielded inactivation of the effect of a suppressor gene in Drosophila melanogaster when eggs in early stages after fertilization were irradiated. A repetition of these experiments with presently available stocks did not confirm the original findings. The data do not support the view that the gene action of Su-er is determined very early, or the alternative view that a substance upon which the gene Su-er acts is inactivated by X rays after fertilization of the egg but before cleavage divisions have begun.

The discovery that X irradiation of Drosophila melanogaster embryos can inactivate the effect of a suppressor gene and thus influence developmental processes that are to unfold several days later was reported by Glass and Plaine in 1950.[1] In the same report it was also shown that irradiation of fertilized eggs, still in meiotic stages, produced similar results, the effect of the suppressor gene having been negated by X rays. Specifically, the experiments involved the gene er (erupt eyes) and its suppressor Su-er. Flies homozygous for er in most cases have large outgrowths of nonfaceted material near the center of one or both eyes, and in some cases several bristles may be present; in weaker manifestations of this character the facets may be merely disarranged, or there may be only an extra bristle at the anterior margin of the eye. When er/er individuals are homozygous for Su-er, the effect of the erupt gene is suppressed almost completely, but occasionally flies may show either the weak or extreme erupt phenotype. The above authors observed that when eggs or larvae from parents homozygous for both er and Su-er were irradiated with 1000 r of X rays, the effect of the suppressor gene was inactivated. As a result, about 90 to 100% of the treated individuals had erupt eyes. Even when eggs only 8±8 minutes old were irradiated, there was 100% inactivation of the Su-er effect, but irradiation of eggs and sperm prior to fertilization failed to cause such inactivation. Assuming that these 8±8-minute-old eggs "could hardly have reached the first cleavage division, on the average," Glass and Plaine concluded that the entrance of the sperm into the egg immediately activates it to produce "at least one specific gene-

initiated substance or morphogenetic system," and that this is inactivated by X rays. During the second and third larval instars, X rays had a progressively diminishing inhibitory effect on the action of Su-er, indicating "either that the primary gene product or substrate is gradually being used up, or that the morphogenetic system in which the product (or substrate) participates has advanced beyond the stage at which the product or substrate can modify it." Since this report, Su-er has been often considered the earliest-acting gene in the development of a multicellular organism.[2] Glass and Plaine stated further that "the target altered in nature by irradiation is probably to be identified as a product of the suppressor gene, ··· ." In 1957, however, the senior author[3] pointed out that an alternative interpretation is possible according to which "the sensitive substance" may be "an essential substrate or precursor for the gene's action," and if the latter is the case then "the action of the suppressor might actually be concurrent with differentiation of the eye."

Recent cytological evidence[4] has shown that, even when females are induced to lay eggs rapidly and collection periods are only 5 minutes, still a certain percentage of eggs are already in cleavage stages at the time of fixation. The mortality rate of irradiated eggs is known to vary with treatment during meiosis and early cleavage stages,[5] but the eclosion frequencies taken from the data of Glass and Plaine do not appear to be what one would expect if their egg samples were homogenous in age when treated and the dose given were 1000 r. Accordingly, it is possible that their samples contained some eggs that might have been retained for several hours after fertilization by the females, and that only these eggs survived and were induced to have the erupt phenotype. It was decided

to repeat their experiments, with special effort being made to accurately time the developmental stages of the treated eggs. If the hypothesis is correct that the effect of the suppressor is not inactivated in very early stages, then the present methods should produce no, or very few, erupt-eyed flies. The results to be reported herein tend to support this expectation.

Materials and Methods. -- Stocks: The flies used in these experiments were kindly supplied by the California Institute of Technology and by Professor Bentley Glass. The first stock, B82, homozygous for the genetic markers Su-er, tu (tumor), bw (brown eyes) on the second chromosome and st (scarlet eyes), er, su-tu (suppressor of tumor) on the third chromosome was used without modification. Stock B92A, Su-er bw; st er originally had attached-X chromosomes and the males carried a genetically marked free X; unmarked and nonattached-X's were substituted for these. The third stock, B91;83, was synthesized by combining Su-er bw from stock 91 with st er from stock 83; this stock also had free and unmarked X chromosomes.

Egg collections and cultures: Virgin females and males were collected and aged separately for 6 to 8 days in vials containing well-yeasted cornmeal-molasses-agar medium. About 30 females and 60 males were stored in their separate vials and were transferred to fresh culture vials two or three times during the aging period. Early in the morning on the day the experiments were to be carried out, the stored females were shaken into the vials with the males. Between 2 and 3 hours later the flies from each vial were shaken into the egg-collecting tubes (Hildreth and Brunt[4]). Eggs collected during the first hour were discarded in order to reduce the number of eggs that might have been retained by the females.

Collection periods for the experiment were from 5 to 30 minutes depending on whether the samples were to be irradiated immediately, aged, or used for controls. After the "immediate" irradiations, eggs were transferred to small squares of water-soaked green blotting paper, lined up for easy counting, and placed in culture vials containing yeasted cornmeal-molasses-agar medium. The control samples and those to be irradiated later were transferred as soon as each collection was terminated. Not more than 50 to 60 eggs were placed in any control or late-irradiation vial to ensure that cultures would be uncrowded, but up to 100 or more were placed in a few immediate-irradiation cultures as the irradiation would prevent the great majority of eggs from hatching. Upon eclosion, the eyes of the adults were examined with the aid of a binocular dissecting microscope at a magnification of 37.5. The eyes were classified as normal, weak erupt, or extreme erupt. The cultures were kept until it was apparent that no more adults would eclose and in no culture did any eclose after the fourteenth day. Some collections were made at room temperature and others at 25°C. All cultures were kept at 25°C.

Irradiations: To eliminate the possibility that the intensity or wave length of X rays used might account for the differences which became apparent between my results and those of Glass and Plaine, it was decided to use three machines, each with different kilovoltage, milliamperage, and filtration. In most experiments an Andrex industrial machine with a Eureka tube having 1.5-mm aluminum inherent filtration was used; operated at 140 kv, 4 ma, with no added filtration at a target distance of 9-1/4 inches, the machine delivered about 105r/min. The second was a Philips X-ray machine with a Philips tube having 1.4-mm aluminum inherent

filtration. This tube delivered about 110 r/min when operated with no added filtration at 60 kv, 15 ma, and at a target distance of 12 inches. The third, a Picker X-ray machine with a Machlett tube having 2-mm beryllium inherent filtration, delivered about 250 r/min at a target distance of 6-3/8 inches when operated at 50 kv, 16 ma, with 1.0-mm aluminum added filtration. The total dose given with any of these machines was 1000 or 1500 r. Irradiations of the immediate and 30-minute groups were terminated late in the afternoon so that even if a female was inseminated as soon as placed with the males, and a fertilized egg was retained until just prior to the last irradiation, such an egg could not have been more than about 8-1/2 hours old when treated.

Cytology: In order to estimate the frequency of eggs that might be in meiosis or in stages prior to the first cleavages when treatment was initiated, eggs were taken from 10-minute collection periods, placed on cover slips, and fixed within the next 8 to 10 minutes. The eggs were then stained by the Feulgen whole-mount procedure[6] and scored as to their stages of development.

Results. -- When eggs from stock B82 were irradiated, the results were quantitatively different from those of Glass and Plaine. Eggs treated when 16 to 21 hours old yielded only about 18% erupt-eyed individuals, with 11% being of the extreme phenotype (TABLE 1). However, when young eggs were irradiated, none of the surviving individuals showed the erupt phenotype. Since Glass and Plaine obtained high yields of extreme erupt when eggs were irradiated during any developmental stage, it was decided to try a second stock derived from one supplied by Professor Glass. This stock, B92A, produced results similar to those from B82. When eggs 10

and 17 to 19-1/2 hours old were irradiated, the yield of individuals with erupt eyes was low (TABLE 1) when compared with the results obtained by the earlier authors, and irradiation of young eggs, even up to 141 minutes old, failed to produce the erupt phenotype in any of the adults.

The stock B91;83 was then synthesized, and the results for eggs irradiated when about 18 hours old were comparable to those of Glass and Plaine (see TABLE 2); they obtained 88% erupt with 65.8% being of the extreme phenotype, while I had a yield of 72.2 to 95% extreme erupt, dependent upon the X-ray machine used. However, when eggs were irradiated immediately after 10-minute collection periods or when approximately 30 minutes old, the frequency of erupt-eyed individuals was comparable with that of the control samples. Thus, irradiation of these early stages did not result in the inactivation of the effect of the suppressor gene.

Amongst the 179 eggs examined cytologically, 129 (72.1%) were fertilized. These 129 included 84 that were in meiosis or in prophase of syngamy and 12 that were undergoing the first cleavage at the time of fixation. Thus, at the maximum, not more than 96 (74.4%) could have been in precleavage stages if irradiation were started immediately after the collections were terminated. Of the remaining eggs 23 were in the second cleavage, 6 in the third, 2 in the fourth, and 2 probably in the seventh or eighth cleavage divisions.[7]

Discussion. -- The immediate problem is to determine the cause for the differences between the results presented herein and those obtained by Glass and Plaine. The first reason that comes to mind is that obviously the stocks used in the present experiments are not the same, and at least 15 years elapsed between the original and repeat tests. The results of

irradiations of the present stocks indicate that there are differences between either the suppressors or the erupt alleles, or that modifiers of either gene have appeared. The fact remains, that irradiation of late egg stages from stock B91; 83 produces the erupt phenotype with high frequency, but irradiation of early stages does not. This might indicate that the suppressor has changed so that its effect can be inactivated in the late but not in the early stages of embryonic development. Alternatively, one might assume that an enhancer of Su-er has appeared in the stock and that this protects the suppressor effect from inactivation by X rays in the early stages but, perhaps because of complete utilization of the enhancer product or inactivation of the enhancer gene during embryonic development, the same protection is not available in later stages. Either of these interpretations may prove to be correct, but unless a stock can be found that will produce results similar to those originally obtained by Glass and Plaine, the alternative explanation presented below appears to be more plausible.

Basing their assumption that the 8-minute embryos "could hardly have reached the first cleavage division, on the average" on the embryological studies of the timing of meiotic and mitotic divisions done by Rabinowitz,[8] Glass and Plaine did not take into account the fact that he used a "highly inbred vigorous Florida stock" and "When an egg was laid later than 5 minutes after the preceding egg deposited by the same female, it was usually discarded." There is no a priori reason to assume that all strains of flies oviposit at the same rate, and it is extremely unlikely that a female lays eggs at 5-minute intervals for a very long period. Even by using vigorous stocks and methods that highly stimulate laying, an average yield of only 3 to 4 eggs per female per hour was obtained over a 2- to 3-hour

period; after this the rate falls off considerably. Females of the Su-er;er stocks tested would not be considered especially rapid layers, and in the present experiments the average was 1.4 eggs per female per hour for those samples irradiated immediately. Of course, this rate includes eggs from the noninseminated females that are also induced to lay rapidly. Also, if the females used in the experiments by Glass and Plaine were placed with males the day or night before the eggs were collected, it would be possible that some were retained for 10 or more hours and that irradiation of these could account for the erupt phenotypes, the young eggs being killed by the treatment. It should be pointed out that the frequencies of eclosion, from egg to adult, in their experiments were similar when eggs were irradiated at 1, 3, and 5 hours (3.2, 2.0, and 3.2%, respectively) and at 10 and 18 hours (15.2% each), and that it was only 6.8% at 15 hours; the number of eggs irradiated at 8 ± 8 minutes is not given. In the present experiments there is only a slight reduction below that of the control rates when eggs were irradiated at 10 or 18 hours. In fact, when eggs from stock B92A were irradiated at 17 to 19-1/2 hours, the eclosion rate was higher than that for the controls; this, undoubtedly, was because of a slightly higher frequency of fertilized eggs in the irradiated than in the control groups. In contrast, in a sample of 3014 eggs irradiated when 1 hour ± 5 minutes old (unpublished data), the eclosion rate was 1/132 that of the control, yet, in the Glass and Plaine experiments, eggs irradiated at a similar stage had an eclosion rate about 1/30 that of their controls. This might be explained if their samples contained some eggs in advanced stages less sensitive to the killing effects of X rays than the early stages.

The difference in wavelength produced by the three X-ray machines had no apparent effect when eggs were irradiated immediately after being collected (TABLES 1 and 2) but the longer wavelengths (TABLE 2) appear to have been more effective than the short ones when eggs were treated during the late developmental stages. The differences in sample size (641 vs 20 adults examined) may account for the discrepany in the results produced by the two machines when 1000 r of X rays were given at 18 hours. The high dose (TABLE 2), of course, had a greater killing effect in both early and late stages, but the frequency of erupt individuals was not increased above that for the control or for the lower dose when young eggs were treated.

Failure to produce the erupt phenotype when Su-er;er eggs were irradiated immediately after fertilization removes support for the theory that gene action of Su-er is determined at this early stage or that a substrate upon which this gene may act is inactivated at this time by X rays. Further experiments are in progress to determine more accurately at which stage the suppressor effect can be inactivated. Discussion of the influence of irradiation on the Su-er;er system will be postponed until this is established.

FOOTNOTES AND REFERENCES

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1. Glass, B., and H. L. Plaine, Proc. Nat. Acad. Sci. 36, 627 (1950).

2. For example, see page 12 in Physiology of Insect Development, ed. F. L. Campbell (Chicago: The University of Chicago Press, 1959).

3. Glass, B., Science, 126, 683 (1957).

4. Hildreth, P. E., and C. Brunt, D. I. S., 36, 128 (1962).

5. Würgler, F. E., H. Ulrich, and A. Schneider-Minder, in Repair from Genetic Damage and Differential Radiosensitivity in Germ Cells, ed. F. H. Sobels (London: Pergamon Press, 1963) p. 101.

6. von Borstel, R. C., and D. L. Lindsley, Stain Technol., 34, 23 (1959).

7. Two of the 84 presyngamy eggs had two sperms, and the rest were monospermic.

8. Rabinowitz, M. J., J. Morphol., 69, 1 (1941).

TABLE 1

Effect of X rays on the Su-er;er system

Age when irrad. started	Number of Eggs	Number of Adults	% Eclosion	% Nonerupt	Extreme erupt Number	Extreme erupt %
Stock B82 Su-er tu bw; st er su-tu						
Control	632	344	54.4	98.8	0	0
$3\frac{1}{2}\pm3\frac{1}{2}$ min	269	5	1.9	100	0	0
16 - 21 hr	499	248	49.7	82.3	27	10.9
Stock B92A Su-er bw; st er						
Control	2086	1326	63.6	97.8	0	0
6±6 min	900	76	8.4	100	0	0
	*501	25	4.9	100	0	0
28 - 90 min	505	0	0			
120 - 141 min	144	4	2.8	100	0	0
10 hr	914	458	50.1	91.5	7	1.5
17 - $19\frac{1}{2}$ hr	618	449	72.7	88.2	34	7.6

*A total dose of 1000 r was given with the Andrex machine except where marked * (Philips machine *). See Materials and Methods for description of output and technique used.

TABLE 2

Effect of X rays on the Su-er;er system

Stock B91;83 Su-er bw;st er

Age when irrad. started	Numbers of Eggs	Numbers of Adults	% Eclosion	% Nonerupt	Extreme erupt Number	Extreme erupt %
Control	2070	1153	55.7	97.1	9	0.8
1000 r						
$5\frac{1}{2}\pm5\frac{1}{2}$ min	3134	133	4.2	97.0	3	2.3
	*626	36	5.8	97.2	0	0
30±5 min	1334	6	0.4	83.3	0	0
18 hrs±15 min	1163	641	55.1	14.7	463	72.2
	*58	20	34.5	0	19	95.0
1500 r						
$5\pm5\frac{1}{2}$ min	197	1	0.5	100	0	0
	*639	3	0.5	100	0	0
18 hrs$\pm5\frac{1}{2}$ min	120	16	13.3	0	16	100
	*160	8	5.0	0	8	100

*See Materials and Methods for description of X-ray machines and the techniques used. The Andrex machine was used except where marked * (Picker machine *).