EFFECTS OF ACTINOMYCIN D ON CROSSING OVER IN *DROSOPHILA MELANOGASTER*¹

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STUDIES on crossing over are usually designed to correlate changes in crossing over values with a specific genetic or environmental condition. The lack of understanding of the primary genetic effects of chromosome rearrangements makes inferences based on the effects of these aberrations on crossing over difficult (SUZUKI 1963b). On the other hand, extrinsic factors (temperature, radiation, chemicals) which affect crossing over result in a number of physiological changes, any of which might be the primary influence on crossing over. The work reported here is a study of the possible role of a specific molecular group in crossing over.

On the basis of studies on the effects of ribonuclease (KAUFMAN, GAY and McELDERRY 1957) and far-red radiation (KAUFMAN *et al.* 1962), it has been concluded that RNA is involved in the process of crossing over. Actinomycin D is an antibiotic which specifically binds to DNA and inhibits synthesis of DNA-mediated RNA (dm-RNA) (REICH 1964). In order to test the role of dm-RNA in crossing over, SUZUKI (1963a) studied the effects of actinomycin D on *Drosophila melanogaster* females. He found a specific effect which is first detected six to nine days after treatment—crossing over was increased only in the region spanning the centromere and all other crossover regions were unaffected. This report is an extension of these observations.

MATERIALS AND METHODS

Crossing over was measured in chromosome 3 using the following markers (numbers are map locations, as listed by BRIDGES and BREHME 1944): *ru*: roughoid, 0.0; *h*: hairy, 26.5; *st*: scarlet, 44.0; *p^p*: pink-peach, 48.0; *ss*: spineless, 58.5; *e^s*: ebony-sooty, 70.7. The region from *ru* to *h* is designated 1, from *h* to *st* 2, and so on. The centromere lies between *st* and *p^p* in region 3.

In order to minimize the background genetic variability, both the Oregon-R and marker stocks were made isogenic by standard use of balancers (*SM-1* and *Ubx*). *F*₁ females, 24 to 48 hours old, were injected in the manner outlined by SUZUKI (1963a). Twenty-four hours after the treatment, the surviving females were individually mated with three males and transferred to fresh vials every three days for five consecutive broods. Control flies were of two types: (1) some females were not injected but were otherwise handled in the same way as the injected flies; (2) the others were injected with a physiological saline solution (0.7N). Actinomycin D was prepared less than 1 hr before each series of injections. It was dissolved in a drop of 95 percent ethanol before the saline solution was added. The concentrations tested were 1.0 µg/ml, 10 µg/ml and 100 µg/ml.

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RESULTS

The crossover data for all treatments except 1.0 $\mu\text{g}/\text{ml}$ actinomycin D are summarized in Table 1. Crossover data from single females were included only if at least two fertile broods were obtained. There is a sudden drop in totals between Broods 3 and 4 in both the saline control and 10 $\mu\text{g}/\text{ml}$ actinomycin D series because progeny counts in some families in these two series were stopped after Brood 3.

With the exception of region 1, crossover values in the injected controls show

TABLE 1

Crossover values in each region of chromosome 3 for each treatment in successive three-day broods

Treatment	Region	Brood number				
		1	2	3	4	5
No injection	Total	710	819	835	636	490
	1	23.8	21.7	23.8	24.5	28.5
	2	20.0	20.5	18.7	17.1	17.8
	3	3.5	3.3	2.4	2.0	2.5
	4	10.1	8.4	6.8	5.0	1.7
	5	14.5	12.2	12.8	13.2	9.9
	Map length	71.9	66.1	64.5	61.8	60.4
0.7N saline	Total	2,867	4,269	4,276	2,847	2,584
	1	22.1	22.4	23.7	24.1	24.7
	2	21.7	19.2	19.2	17.3	15.6
	3	4.6	3.8	3.6	2.2	1.3
	4	11.0	8.3	7.7	8.1	6.4
	5	12.0	12.2	9.3	11.8	11.7
	Map length	71.4	65.9	63.5	63.5	59.7
10 $\mu\text{g}/\text{ml}$ actinomycin D	Total	1,446	3,234	3,058	2,558	1,996
	1	21.4	21.7	20.2	19.2	21.3
	2	19.5	19.3	18.7	18.8	19.4
	3	4.7	3.4	6.3	6.1	5.2
	4	9.8	8.2	8.5	9.1	9.0
	5	12.9	11.3	10.9	12.6	11.9
	Map length	68.3	63.9	64.6	65.8	66.8
100 $\mu\text{g}/\text{ml}$ actinomycin D	Total	357	658	1,038	891	955
	1	22.7	22.7	19.3	18.7	20.0
	2	18.8	20.5	19.1	20.4	20.1
	3	4.8	4.2	9.7	13.8	7.5
	4	12.3	8.3	10.6	10.4	10.1
	5	14.3	11.8	11.8	12.5	12.9
	Map length	72.9	67.5	70.5	75.8	70.6

a gradual decrease with increasing age of the mother, as BRIDGES (1927) first noted. Crossover values in each region of the uninjected females were compared with the corresponding values in the saline-injected flies in all five broods. Only three of the 25 comparisons showed significant differences: region 5 of the saline-injected females in Brood 3 was lower than in the uninjected and region 4 in Broods 4 and 5 was higher in the saline-injected than in the uninjected flies. It can be concluded that injection of physiological saline does not interfere significantly with normal crossover events. However, all further comparisons will use the saline injection data as controls.

In the 1.0 $\mu\text{g}/\text{ml}$ actinomycin D series, crossing over was measured in region 3 and in five females only. The following numbers were obtained:

	Brood number				
	1	2	3	4	5
Total progeny	84	331	303	437	348
Crossover percent	6.0	4.2	1.6	2.3	0.9

None of these crossover values is significantly different from those obtained from saline-injected controls which are listed in Table 1. In both the 10 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ actinomycin D series, crossing over in region 3 was significantly higher than in the control series for Broods 3, 4 and 5 (Table 1).

The ratios of crossover values for each region in the actinomycin D series (p_1) to the values in the controls (p_0) for the same region of each brood are plotted in Figure 1. The method used to calculate the 95 percent confidence limits of each

FIGURE 1.—Ratio of crossover values in each region of chromosome 3 in actinomycin D-injected females to their respective control values in each brood.

ratio has been outlined previously (SUZUKI 1962). Any ratio and its confidence limits which spans the value of 1.0 indicates that the two values compared are not significantly different.

It can be seen (Figure 1) that actinomycin D has no effect on crossing over in any region in the first two broods. In Brood 3 a striking increase in crossing over in region 3 occurs and the relative increase rises further in Broods 4 and 5. This differs from the preliminary observation (SUZUKI 1963a) that the increase was maximal in Brood 3. Significant differences from control values are also induced in the other regions. Crossing over in region 1 appears to be depressed by actinomycin D treatment whereas values for regions 2, 4 and 5 are increased slightly. The maximal effect of actinomycin D on the centromere region and the smaller, but significant effects on the adjacent regions are strikingly similar to the interchromosomal effects of chromosome rearrangements (SUZUKI 1962, 1963b) and the possible significance of this similarity will be discussed.

The distribution frequency of females having different crossover values (where the numbers on the abscissa represent values in the interval between the number and the next higher integer) in region 3 is plotted broodwise for the saline and 10 $\mu\text{g}/\text{ml}$ actinomycin D series in Figure 2. Data from females treated with 100 $\mu\text{g}/\text{ml}$ actinomycin D are not plotted owing to the small number of fertile females in each brood. In Broods 1 and 2 the distributions of females in each crossover class after actinomycin D and saline treatment are similar. In the third and subsequent broods, actinomycin D induces an increase in the frequency and values of the higher crossover classes represented.

FIGURE 2.—Distribution frequency of females with values in each crossover percentage class. The arrows indicate the position of the means for each series.

FIGURE 3.—Cumulative distribution frequency of females with values in each crossover percentage class.

The change in relative proportions of females having higher crossover values can be seen in the cumulative distribution frequencies plotted in Figure 3. In Brood 3, more than 20 percent of the females treated with actinomycin D have crossover values greater than the highest crossover percentage class represented in the control series. In Brood 4, 30 percent of the actinomycin D-treated females have crossover values higher than 8.0 whereas less than 2 percent of the controls have a higher value; 56 percent of the actinomycin D series have values larger than 4.0 whereas 16 percent of the controls have values higher. 50 percent of the actinomycin D-treated females in Brood 5 have values greater than 4.0 whereas less than 5 percent of the controls have values higher. Thus, the proportion of cells in which increases in crossing over are induced by actinomycin D is greater in cells which are presumed to be in gonial stages at the time of treatment. THOMPSON (1964) and R. GRELL (personal communication) have found that temperature also increases crossing over in gonial cells.

The effectiveness of actinomycin D in increasing crossing over is striking if data from females sterile in Broods 1 and/or 2 are analyzed. Only three of the saline-injected females (except those completely sterile) were sterile in Brood 1. On the other hand, half of the females injected with 100 $\mu\text{g}/\text{ml}$ actinomycin D which produced progeny were sterile in Broods 1 and 2. It can be seen that crossing over in region 3 was greatly increased in such females (Table 2). After treatment with 10 $\mu\text{g}/\text{ml}$ actinomycin D the three females which were sterile in

TABLE 2

Crossover values in region 3 in females sterile in the first brood(s)

Actinomycin D concentration	Brood Number				
	1	2	3	4	5
100 μ g/ml	sterile	sterile	21.6 (231)*	24.3 (304)	10.6 (411)
10 μ g/ml	sterile	sterile	20.5 (73)	22.6 (168)	21.4 (140)
10 μ g/ml	sterile	3.4 (631)	12.9 (613)	7.7 (617)	7.1 (600)
0 (control)	sterile	2.9 (170)	2.2 (184)	5.3 (208)	4.2 (142)

* Total number of offspring is given in parentheses.

Broods 1 and 2 had very high crossover values, and the 11 sterile in Brood 1 also had increases above the brood means for the entire treated series, in Broods 3, 4 and 5 (Table 2). If sterility induced in the first broods is used as an indication of uptake of the antibiotic into the germinal tissue, it can be seen that actinomycin D is much more potent in its effect on crossing over than the series totals indicate.

The high concentrations of actinomycin D were used in order to overcome the presumed dilution of the chemical in the egg cytoplasm and by the action of the nurse cells. Thus, whereas 1.0 μ g/ml of actinomycin D has no effect on crossing over, viability or sterility in injected females, the same concentration causes high mortality and sterility as well as increased radiosensitivity when injected into males (MUKHERJEE and SUZUKI 1964). R. VALENCIA (personal communication) has noted that chemicals which are mutagenic in males have no effect when injected into females at the same concentration. The factor of dilution would seem a logical explanation. In the absence of a biochemical assay for the activity of actinomycin D, mortality and sterility induced by injection were used as biological criteria.

The effects of the treatments on survival are shown in Figure 4. Injection of saline results in an initial mortality of 10 percent presumably caused by mechanical injury during the injection process. Injection of actinomycin D at a concentration of 10 μ g/ml increases the initial mortality to 20 percent and thereafter viability of the survivors is similar to that of the controls. A concentration of 100 μ g/ml of actinomycin D results in a continued mortality through the first broods so that about 70 percent of the flies die within four days of injection. By the criterion of mortality then, actinomycin D is biologically effective and the degree of its effectiveness is concentration dependent.

TABLE 3

Percent of the survivors in each treatment which were completely sterile

Treatment	Total injected	Survivors in Brood 5	Survivors completely sterile	
			Number	Percent
No injection	16	16	2	12.5
0.7% NaCl	99	77	10	12.9
10 μ g/ml actinomycin D	111	70	13	18.5
100 μ g/ml actinomycin D	158	37	16	43.2

FIGURE 4.—Percent survival in each treatment at three-day intervals.

Table 3 indicates the effects of injection on the induction of complete sterility. Saline injection alone does not increase the proportion of individuals which is sterile in all five broods. Actinomycin D does induce sterility, and at $100\text{ }\mu\text{g/ml}$, over 40 percent of the surviving flies are completely sterile. The criterion of sterility also indicates a potent biological effect of actinomycin D.

The average number of offspring per fertile female in each brood is shown in Table 4. The apparent increase in offspring in saline-injected females relative to the uninjected controls could be spurious. The average number of offspring for each brood is calculated only on the number of females giving fertile cultures in that brood. Since the data from females sterile in one or two broods are included in the progeny total, if the sterility was due to an inability to lay eggs in a particular brood, these unlaid eggs together with the subsequently maturing batch could increase the average number in the fertile brood. It can be seen that at $10\text{ }\mu\text{g/ml}$, actinomycin D does not affect the average number of progeny per

TABLE 4

Average number of offspring per fertile female in each brood of each treatment

Treatment	Brood Number					Total per female
	1	2	3	4	5	
No injection	54.6	58.5	59.6	45.4	37.7	255.8
0.7N NaCl	48.6	70.0	70.1	52.7	47.9	289.3
10 $\mu\text{g/ml}$ actinomycin D	35.3	70.3	66.5	56.8	45.4	274.3
100 $\mu\text{g/ml}$ actinomycin D	35.7	59.8	54.6	46.9	53.1	250.1

fertile female whereas at 100 $\mu\text{g}/\text{ml}$ there is a reduction in progeny (Table 4). The absence of a striking decrease in progeny after actinomycin D-treatment indicates that the increase in tetrads of rank 2 and 3 at the expense of rank 0 and 1 tetrads reflects a real increase in crossing over.

DISCUSSION

The preliminary report of an effect of actinomycin D on crossing over (SUZUKI 1963a) has been confirmed by these experiments and by P. A. THOMPSON (personal communication). The use of physiological saline for injection into control females and for actinomycin D solutions instead of distilled water as in the earlier report, obviates the problems of induced sterility and possible cell selection by osmotic shock. When compared with uninjected females, by the criteria of induced sterility, average number of progeny per female, and crossover values, saline injection appears to have no biological effect. Actinomycin D, on the other hand, does have physiological effects (indicated by increases in sterility and mortality) and genetic effects (indicated by changes in crossover values) at higher concentrations. Average progeny produced per fertile female and tetrad analyses do not indicate selective elimination of the products of low rank tetrads which could result in an apparent increase in crossover frequencies. The effect of actinomycin D then, must result from an effect on meiotic and premeiotic events leading to a crossover.

Numerous studies on the mode of action of actinomycin D clearly indicate that its primary physiological effect at lower concentrations is very specific, namely the inhibition of all DNA-mediated RNA synthesis; at higher concentrations, both RNA synthesis and DNA replication are inhibited (see REICH 1964 for a review). Moreover, actinomycin D appears to be universally effective, having been studied in microbial systems (REICH *et al.* 1961), plants (HOTTA and STERN 1963), amphibians (IZAWA, ALLFREY and MIRSKY 1963), mammals (FRANKLIN 1963), and Diptera (BEERMANN 1963). An inhibition of DNA replication by actinomycin D would be expected to decrease the number of offspring recovered per female in Broods 3, 4 and 5. Since there is no evidence of such a reduction in females injected with a 10 $\mu\text{g}/\text{ml}$ concentration (Table 4), the inhibition of dm-RNA synthesis by actinomycin D is probably responsible for the physiological and genetic effects noted in this report. Survival of the germ cells under actinomycin D treatment is probably due to large cytoplasmic reserves in the egg and its massive nourishment by the surrounding nurse cells.

Assuming that the primary effect of actinomycin D when injected into *Drosophila* females is the inhibition of DNA-mediated RNA synthesis, SUZUKI (1963a) has considered a few of the simplest possible explanations which might account for the observed increase in crossing over. He has favored the hypothesis that the region around the centromere of chromosome 3 is actively functioning at the time of crossing over, that actinomycin D interferes with this functioning, and that the subsequent inactivation results in an alteration of the chromosomal structure in that region thereby rendering exchange more likely (possibly by permitting more intimate synapsis). Evidence for this model comes from the observation that inhibition of dm-RNA synthesis by actinomycin D results in the

collapse of loops in amphibian lampbrush chromosomes (IZAWA, ALLFREY and MIRSKY 1963) and of puffs in salivary gland chromosomes (BEERMANN 1963; LAUFER, NAKASE and VANDERBERG 1964).

Region 3 spans the centromere and thus includes the proximal heterochromatin of chromosome 3. The nucleolus-organizing region of chromosomes, which is typically heterochromatic, has been shown to synthesize ribosomal RNA (PERRY 1962; McCONKEY and HOPKINS 1964). One may suggest that proximal heterochromatin in *Drosophila* bears genetic information for the synthesis of molecules such as ribosomal or transfer RNA, or for the production of activating enzymes. Although BAKER (1958) has evidence that crossing over does not occur in heterochromatin, the postulated effect of the structural configuration of functioning heterochromatin could also extend into adjacent euchromatic segments. DOBZHANSKY (1934) noted that suppression in crossing over between X chromosomes by the presence of duplications extended far beyond the ends of the duplications. The hypothesis outlined could be extended to gonial crossing over as well. STERN (1936) and WALEN (1964) have shown that somatic crossing over in *Drosophila* occurs primarily in proximal heterochromatin; actinomycin D could enhance this by the proposed scheme.

In a comparative study of the effects of radiation, actinomycin D and a reversed acrocentric compound X chromosome on crossing over in three subregions of region 3, SUZUKI and PARRY (1964) noted that these agents have qualitatively similar effects. They suggest that these experimental conditions are not acting directly on the crossover process itself. The similarity in pattern of effect of actinomycin D-treatment to the interchromosomal effects of rearrangements (SUZUKI 1962, 1963b) could suggest that extrinsic or intrinsic conditions which result in increases in crossing over may create a physiological state which inactivates chromosome regions at the time of crossing over.

One final point might be noted. Recent evidence in microbial genetics suggests that recombination between phages (KOZINSKI, KOZINSKI and SHANNON 1963; MEAD and VOLKIN 1964) and in bacteria (TOMASZ and HOTCHKISS 1964; HOWARD-FLANDERS and BOYCE 1964) may be mediated by enzymes. If crossing over in higher organisms is also controlled by one or more enzymes (SUZUKI 1964), the inhibition of dm-RNA synthesis by actinomycin D should result in a decrease in the amount of crossover enzymes, which in turn would result in reduced crossover frequencies. Such a decrease does not occur (Figure 1), and if a crossover enzyme is postulated, further assumptions will have to be made. The hypothesis discussed in this paper to explain the effects of actinomycin D on crossing over represents one of several possibilities. It is sufficiently specific to suggest further critical experiments and it provides an explanation for some genetic puzzles (the apparent inertness of heterochromatin, the disparity between genetic and cytological map distances) but at this time is little more than an intriguing testable hypothesis.

SUMMARY

Crossing over in chromosome 3 was measured in females which were uninjected, injected with a 0.7N saline solution, or injected with actinomycin D at

concentrations of 1.0, 10.0, or 100 $\mu\text{g/ml}$. The females were mated singly and allowed to lay eggs for five consecutive three-day broods.

Crossover values, average number of progeny per brood per female and frequency of complete sterility were not significantly different between saline injected and uninjected females.

Actinomycin D at a concentration of 1.0 $\mu\text{g/ml}$ does not appear to influence crossing over. At 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, actinomycin D increases the mortality of injected females, the frequency of complete sterility of survivors and crossover values in the region spanning the centromere. The increase in crossing over is induced in meiotic and gonial cells and does not appear to be due to cell selection by elimination of lower rank tetrads. The degree of effect of actinomycin D is dependent on the concentration injected.

It is postulated that the active functioning of a gene results in structural properties of that locus that prevent crossing over from occurring in that region. Extrinsic and intrinsic factors may inactivate loci and the consequent structural modifications permit crossing over to occur more frequently.

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