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ACTINOMYCIN D-INDUCED PHENOCOPIES IN *DROSOPHILA MELANOGASTER* AND THEIR RELEVANCE TO DIFFERENTIAL GENE TRANSCRIPTION

THE GENETIC BASIS OF ACTINOMYCIN D-INDUCED PREFERENTIAL KILLING OF *DROSOPHILA MELANOGASTER* MALES

by

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INTRODUCTION

Of paramount importance for understanding the molecular basis of cell differentiation is the increasing experimental evidence that genetic information is transcribed differentially during development. Changes in the populations of RNA transcripts have been demonstrated during morphogenesis in the unicellular bacterial system of *Bacillus subtilis* (Doi and Igarashi 1964) as well as in such multicellular organisms as the sea urchin (Whitely *et al.* 1966; Glisin *et al.* 1966), the frog (Bachvarova *et al.* 1966; Denis 1966; Crippa *et al.* 1967), and the milkweed bug (Harris and Forrest 1967). The changing patterns of puffs in giant chromosomes during the development of such dipteran insects as Drosophila (Ashburner 1967) and Chironomus (Beermann and Clever 1964; Clever and Romball 1966) also provide evidence for differential gene transcription. In Chironomus, it has been shown that puffs are sites of RNA synthesis and that the base composition of RNA varies in different puffs. Actinomycin D causes the inhibition of RNA synthesis, as well as the disappearance of certain puffs.

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Since the apparently major mechanism of action of actinomycin D -- the inhibition of DNA-dependent RNA synthesis -- is well established (Reich and Goldberg 1964), one can also use this antibiotic to gain evidence about differential gene transcription by studying its effect at different stages of development on the morphological traits of a multicellular organism. In the snail Ilyanassa obsoleta, the inhibition of RNA synthesis by actinomycin D has been correlated with the inhibition of differentiation of particular organs at specific stages of embryonic development (Collier 1966). The potential success of actinomycin D as a phenocopying agent in Drosophila is also suggested by the evidence that this antibiotic affects the development of sea urchin embryos (Gross and Cousineau 1963, 1964; Infante and Nemer 1967), amphibian and chick embryos (Brachet and Denis 1963), and Chironomus larvae (Laufer et al., 1964). Although various physical and chemical agents have been used previously to produce phenocopies in Drosophila (see Muller 1939 and Herskowitz 1950, 1957, 1964 for references), these phenocopy-inducing agents do not reveal differential gene transcription because their mode of action is unknown.

The experiments reported here were designed to determine in Drosophila melanogaster the frequency and the pattern of phenocopies of adult traits after larvae -- cultured in a germ-free, chemically-defined medium -- are treated at different stages of development with actinomycin D. The results will be discussed relative to whether differential gene action occurs during the differentiation of imaginal buds. Some of the results have already been reported briefly (Margulies 1967).

MATERIALS AND METHODS

The following stocks and genotypes were used: (1) +/+ ♀♀ × +/Y ♂♂ (Oregon-R, wild-type stock). (2) M/sc^{S1} bb^{1} B In49 oc ptg sc^{8} ♀♀ × sc^{S1} bb^{1} B In49 oc ptg sc^{8}/Y ♂♂. This self-maintaining stock, with all markers X-linked, is henceforth referred to, for short, as M/bb^{1} B oc ♀♀ × bb^{1} B oc/Y ♂♂. M = Minute bristles; sc^{S1} sc^{8} = scute inversion; bb^{1} = bobbed bristles, lethal; B = Bar eye; In49 = inversion 49; oc = ocelliless; ptg = pentagon; (3) M/sc^{S1} B InS w^{a} sc^{8} ♀♀ × sc^{S1} B InS w^{a} sc^{8}/Y ♂♂. This stock is henceforth referred to as M/Basc ♀♀ × Basc/Y ♂♂. w^{a} = apricot eye color; InS = inversion S. (4) +/+ ♀♀ × w.Y^{S}/Y^{Lc} ♂♂. w = white eye color; Y^{S} = shorter arm of Y chromosome, here attached to the X chromosome, believed to contain the nucleolus organizer; Y^{Lc} = longer arm of Y, in ring form.

Eggs laid in a two-hour period on a sugar-agar medium seeded with dead yeast were collected and surface sterilized using Geer's method (Geer 1963). The sterilization of eggs was carried out starting 19-22 hours after the egg laying had begun, at which time all larvae that had hatched from older eggs were discarded. By insuring that the eggs retained were close in age, this procedure provided larvae more uniform in age. (In addition, other precautions were taken to obtain eggs close in age: females were relatively young, usually 3-10 days in age, and flies were placed on fresh food 2-3 days before egg laying to minimize the holding of developing eggs by females; eggs laid one hour prior to the desired two-hour period were discarded in order to eliminate those eggs laid in an advanced stage of development). The sterilization of eggs, as well as all subsequent axenic transfers of eggs and larvae, were

performed in a hood which had been made germ-free by exposure to ultraviolet light. Sterilized eggs were transferred with a calibrated micro-pipette to petri dishes containing about 10 ml of chemically-defined Geer's medium (Geer 1965). Although the number of eggs transferred to a petri dish averaged 90-140 in different experiments, the number of eggs per dish did not vary by more than 20 percent within an experiment.

Two different methods were used for treating larvae with actinomycin D (supplied through the courtesy of Merck, Sharp and Dohme). The first method was used in all experiments except those involving stock 2. Actinomycin D was dissolved in a few ml of ethanol and water, sterilized by passage through a millipore filter, and added to liquid Geer's medium at 60°C. Known numbers of larvae of a given age (stated as the number of days after hatching) were transferred axenically to new petri dishes containing the actinomycin D medium and allowed to feed on it for one day (usually 22-23 hours). Since the eggs collected were found to hatch as larvae over, approximately, a 4-hour period, and the larval transfer required 1-4 hours, the ages of the larvae in a population could have varied by 4 ± 4 hours at the beginning of treatment. After the treatment the number of dead larvae was recorded and all living larvae were transferred to new petri dishes containing sterile standard Drosophila medium to provide favorable conditions for survival and the reversibility of the actinomycin D-DNA complex. Cultures contaminated by fungi or bacteria were detected by subculturing from each of these petri dishes on suitable media. Contaminated cultures (usually 5-10%) were discarded; sterile cultures were kept axenic until the eclosion of the first flies. Control larvae were treated exactly

as experimentals, except that distilled water was substituted for actinomycin D solution. The cultures were kept at $24^{\circ} \pm 0.5^{\circ}C$. Since some of the larvae crawled to the top of the petri dishes during treatment and many of these died, the population size (used to calculate the percentage of eclosion in these experiments) is the number of larvae transferred after treatment plus the number found dead in the medium. In many experiments some untreated larvae were left on Geer's medium to determine the time of pupation.

The second method of treatment differed only in that actinomycin D solution was pipetted directly into the petri dishes containing Geer's medium in which larvae had hatched and developed, and the larvae were transferred after 18-21 hours. In these experiments, because the original number of larvae treated and the number dying during the period of treatment were not determined, the population size (used to calculate the percentage of eclosion) is the number of larvae transferred after treatment. (The eclosion percentage obtained, here especially, is an overestimate which is used to show the relative survival of larvae treated at different ages). The concentration of actinomycin D used ranged from 5-50 μg/ml and depended on the stock and larval age at the time of treatment (1-10 days old). All flies which eclosed before the emergence of the next generation were examined under a standard binocular dissecting microscope.

RESULTS AND DISCUSSION OF SPECIFIC EXPERIMENTS

General phenocopy and mortality effects of actinomycin D: The effects of treating wild-type larvae of a given age with different concentrations of actinomycin D are summarized in Table 1 and Figure 1. Treatment of 8-day old larvae with 10, 20 or 50 µg/ml actinomycin D is evaluated in terms of the effect on total frequency of phenocopies and on viability. The antibiotic clearly induced phenocopies in experiment B, since the percentage of abnormal flies after treatment with 20 or 50 µg/ml is significantly different from the control, the effect increasing strikingly with the higher concentration. With all three concentrations, a significant reduction occurred in the percentage of eclosion, the degree of lethality increasing greatly with the highest concentration. Although 10 µg/ml did not detectably induce phenocopies in experiment A, a 22 percent decrease occurred in eclosion as compared to the control. This suggestive independence of the phenocopy-inducing effect and the mortality effect of actinomycin D is confirmed in subsequent experiments.

The phenocopy and mortality effects of treating wild-type larvae of different ages with actinomycin D are summarized in Table 2. Because young larvae are more sensitive to the killing effect of actinomycin D, different concentrations were used to treat larvae of different ages. Varying the concentration allowed a sufficiently large proportion of the treated individuals to survive, regardless of age when treated, permitting a comparison of the relative susceptibility of different stages of larval development to the induction of phenocopies. Only experiments where eclosion after treatment was more than 8 percent

TABLE 1

Effects of treating 8-day old wild-type larvae with different concentrations of actinomycin D. O = Control.

Experiment	Act. D µg/ml	No. Larvae	No. Eclosing	% Eclosion	% Abnormal Adults*	% Abnormal ♀♀*	% Abnormal ♂♂*
A	0	449	405	90.2	2.2	1.9	2.6
	10	477	335	70.2**	4.8	4.1	5.8
B	0	420	350	83.3	1.4	2.0	0.6
	20	608	415	68.2**	6.5**	6.3	6.7
	50	548	68	12.4**	32.4**	32.5	32.1

* $100 \times \frac{\text{No. abnormal}}{\text{Total eclosed of appropriate sex}}$

** Significant at the 1% level.

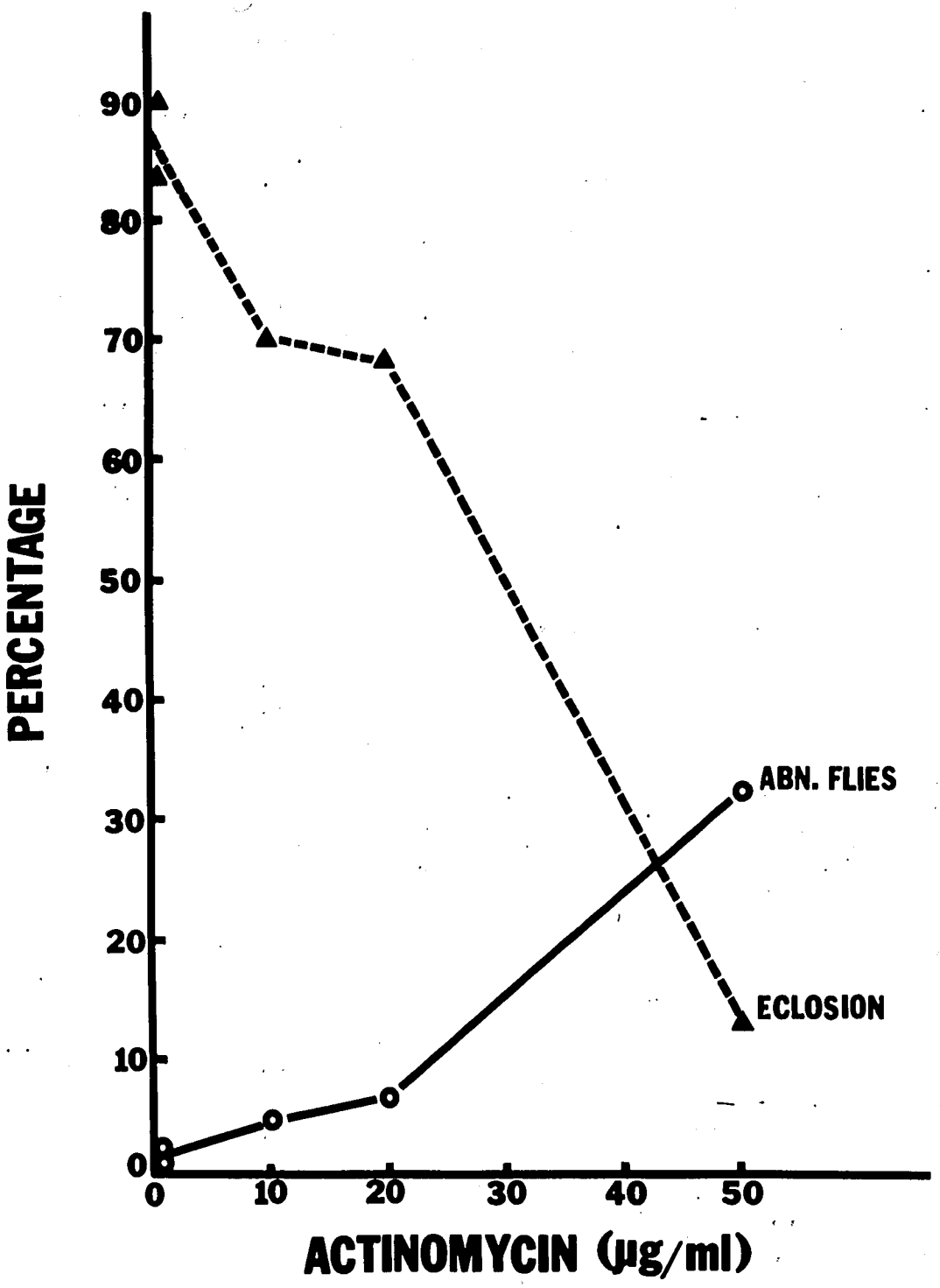

Figure 1. - Effect on phenocopy induction and viability of treating 8-day old wild-type larvae with different concentrations of actinomycin D. Abn. = Abnormal. Data from Table 1.

TABLE 2

Effects of treating wild-type larvae of different ages with different concentrations of actinomycin D. 0 = Control. (Statistical comparisons are made of percent Abnormal Adults in treated and control series, and of percent Abnormal ♀♀ and ♂♂ after different treatments)

Experiment	Act. D µg/ml	Age of Larvae (days)	No. Larvae	No. Eclosing	% Eclosion	% Abnormal Adults†	% Abnormal ♀♀†	% Abnormal ♂♂†
1	0	1	478	432	90.4	3.0	1.9	4.1
	20	1	1400	301	21.5*	3.3	4.4	2.4
	40	1	492	0	0 .0*			
	0	3	166	156	94.0	1.3	2.5	0.0
	20	3	879	669	76.1*	3.3	3.6	2.9
	40	3	678	11	1.6*	9.1	0.0	14.3
	0	5	273	241	88.3	1.2	0.8	1.6
	20	5	588	175	29.8	5.7**	8.2	3.3
	40	5	1025	78	7.6*	17.9*	25.8	12.8
	0	6	271	244	90.0	2.1	1.6	2.6

	0	6	271	244	90.0	2.1	1.6	2.6
	20	6	560	277	49.5*	7.6*	11.3**	4.2
	40	6	833	93	11.2*	17.2*	16.3	18.2
2	0	6	136	123	90.4	1.6	1.9	1.4
	40	6	1300	327	25.2*	17.1*	14.0	20.2
	0	8	223	206	92.4	1.9	1.8	2.2
	40	8	1178	512	43.5*	62.3*	60.7	64.0
	0	10	114	97	85.1	4.1	2.2	5.9
	40	10	593	155	26.1*	72.9*	70.1	76.6
3	0	2	155	135	87.1	3.0	2.8	3.1
	30	2	1540	25	1.6*	20.0**	33.3	12.5
	0	6	319	279	87.5	2.9	2.6	3.1
	30	6	744	157	21.1*	28.6*	28.8	29.7
4	0	4	315	265	84.1	3.0	3.3	2.8
	30	4	1800	281	15.6*	7.8**	6.5	11.1
	0	6	234	208	88.8	10.1	9.7	10.5
	30	6	1087	399	36.7*	19.3*	18.9	19.3

† $100 \times \frac{\text{No. abnormal}}{\text{Total eclosed of appropriate sex}}$

* Significant at the 1% level

** Significant at the 5% level

of the control value are used for comparison of the phenocopy-inducing effect of actinomycin D on larvae of different ages. To avoid the assumption that the lower concentrations which allowed enough survival when young larvae were treated were also high enough to induce phenocopies, 6-day old larvae were ordinarily also tested for the phenocopy-inducing action of a given concentration of actinomycin D whenever larvae of any younger age were treated. Thus, a negative phenocopy effect on 6-day old larvae would indicate an unsatisfactory test of any younger larvae treated in the same experiment. Data for larvae of the same age in different experiments are not combined, since the starting time of pupation was variable, indicating that larvae of the same age in days were not always at the same hour of development in different experiments. This variability is indicated by the different eclosion percentages resulting from treatment of 6-day old larvae with 40 µg/ml in Experiments 1 and 2. In this case, pupation in the control left on Geer's medium started on day 10 in Experiment 1 and on day 9 in Experiment 2. The control 8-day old larvae in Experiment 2 are not equivalent in age to those in Experiments A and B (Table 1), where development was slower (perhaps because of an inferior medium) and pupation started on day 12; this developmental difference probably accounts for the lower phenocopy and eclosion values in the latter experiments.

A comparison of the effects of 40 µg/ml actinomycin D on larvae of different ages (Table 2) is illustrated in Figure 2. The percentage of flies with phenocopies is significantly increased over the control frequency when 5-day old larvae are treated, generally increases with age at time of treatment and reaches the highest frequency of 73 percent for treated 10-day old larvae, the stage closest to pupation.

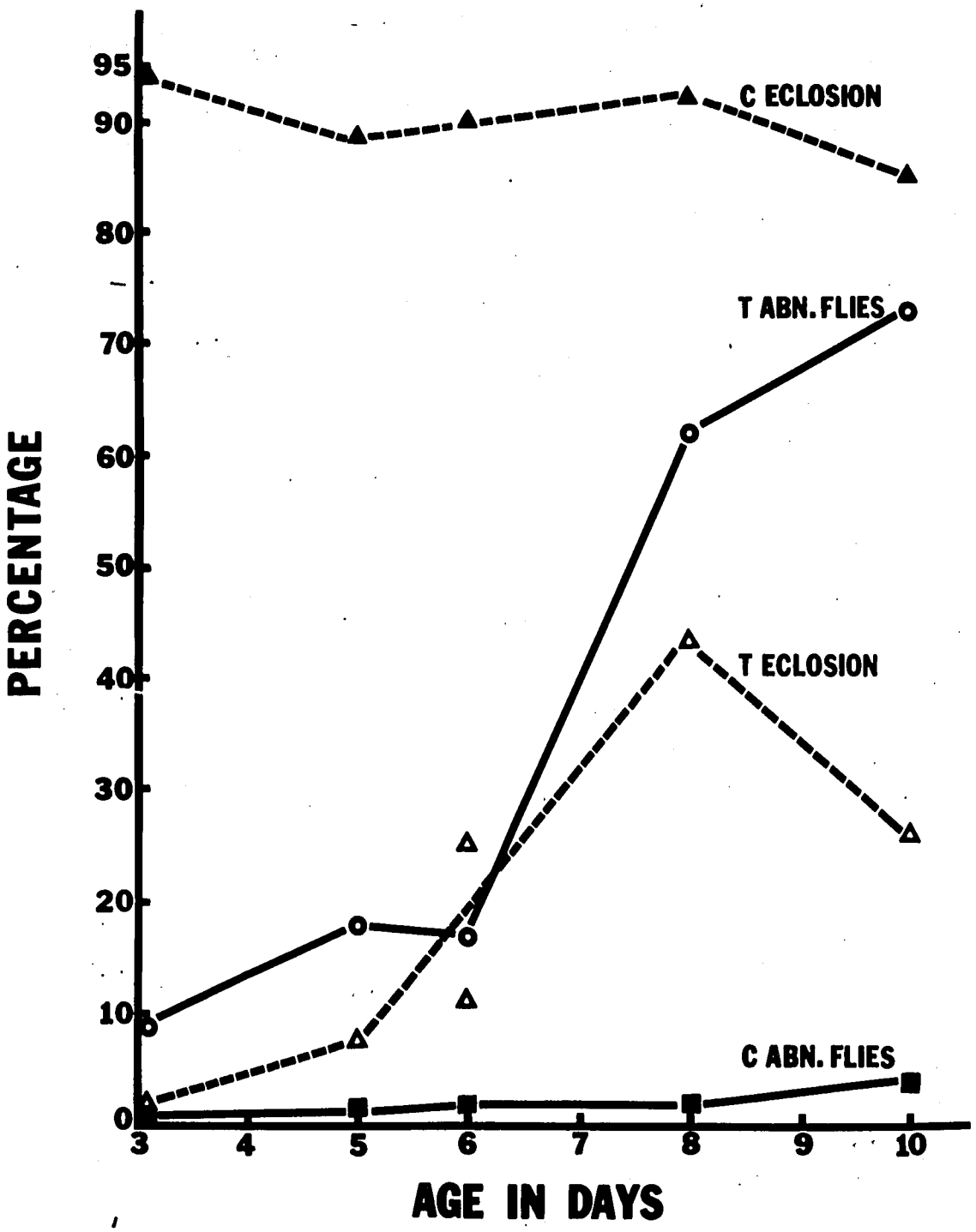

Figure 2. - Effect on phenocopy induction and viability of treating wild-type larvae of different ages with 40 µg/ml actinomycin D. T = Treated series. C = Control series. Abn. = Abnormal. Data taken from Table 2.

(The effect on 3-day old larvae cannot be evaluated because of the very low percentage of eclosion). Eclosion percentage also generally increases with age of treatment, but drops in treated 10-day old larvae, probably because of the critical nature of this developmental period. This decrease in eclosion percentage may be due, partly, to the occurrence of many lethal phenocopies per individual in the pupal stage. This multi-phenocopy explanation is inferred from the observation that treatment of larvae close to pupation (i.e., on days 8 and 10) results both in larval death and arrested development in pupal stages, whereas treatment of younger larvae kills, primarily, in the larval stage. The effects of 40 µg/ml actinomycin D contrast sharply with controls where eclosion ranges from 85-95 percent and relatively very few flies have abnormalities.

Treatment with 30 µg/ml actinomycin D (Table 2) results in very poor survival of 2-day old larvae. Although 5 flies were abnormal (2 of which showed an effect which is not uncommon in control flies), this result cannot be evaluated because of the low eclosion percentage. When 4-day old larvae are treated, however, the eclosion percentage is suitable (15%) and a significant but low frequency of phenocopies is induced. The incidence of phenocopies increases significantly (to 19%) when 6-day old larvae are treated with this concentration.

Treatment with 20 µg/ml actinomycin D (Table 2) of 5-day and 6-day old larvae induced a significant, although small, increase in phenocopy frequency, whereas treatment of 1-day and 3-day old larvae had no detectable effect. (The eclosion percentage obtained after treatment of 3-day old larvae is the only exception to the expectation that younger larvae have a higher mortality than older ones).

It is noteworthy that in 13 suitable comparisons (Tables 1 and 2) only one (6-day old larvae treated with 20 µg/ml) showed a significant difference in the frequency of phenocopies between the sexes.

To determine the effects of actinomycin D on flies of a different genotype, larvae of a multiple mutant stock (stock 2) were treated at different ages. Since the mutant stock was more sensitive to the killing effect of actinomycin D than wild-type, lower concentrations (5 and 10 µg/ml) were used. The data obtained from five experiments performed over a short period of time are summarized in Table 3 and Figure 3. Note that larvae of a given age in the wild-type stock and stock 2 may not represent the same developmental stage, at least in the case of females, since Minutes are well known to retard development. Stock 2 control females eclose at least one day later than males. The general effects on the percentage of abnormal flies and of eclosion are similar to those obtained in the case of the wild-type stock when larvae of different ages are treated with 10 µg/ml actinomycin D. The phenocopy effect with treated, 2-day old larvae cannot be evaluated because of the low eclosion percentage. Phenocopy incidence is not significantly increased over the control by treating 3-day old larvae; it is increased by treating 4-day old or older larvae, rising to 30 percent in the oldest (9-day old). The control eclosion percentage is lower in the mutant than in the wild-type stock, doubtless due in part to the general weakening caused by the markers carried.

Treatment with 5 µg/ml actinomycin D, which permits enough survival of 1-day and 2-day old larvae, does not result in detectable phenocopy induction. This concentration does, however, produce a significant

TABLE 3

Effects of treating stock 2 larvae of different ages with different concentrations of actinomycin D. Cross: M/bb[1] B oc × bb[1] B oc/Y. 0 = Control

Experiment	Act. D µg/ml	Age of Larvae (days)	No.† Larvae	No. Eclosing	% Eclosion	% Abnormal Adults‡	% Abnormal ♀♀‡	% Abnormal ♂♂‡	Adult Sex Ratio ♀♀/♂♂
5	0	1	162	101	62.3	3.0	4.9	0.0	1.5
	5	1	308	70	22.7*	2.8	2.6	3.2	1.3
	10	1	468	2	0.4*	0.0	0.0	0.0	--
6	0	2	350	203	58.0	3.4	4.2	2.4	1.4
	5	2	665	176	26.8*	4.5	7.9	0.0	1.3
	10	2	418	11	2.6*	0.0	0.0	0.0	--
7	0	3	331	178	53.7	3.3	6.1	0.0	1.3
	5	3	433	262	60.5	2.3	3.6	0.8	1.1
	10	3	843	126	14.9*	7.9	8.9	5.6	2.5*

6	0	2	350	203	58.0	3.4	4.2	2.4	1.4
	5	2	665	176	26.8*	4.5	7.9	0.0	1.3
	10	2	418	11	2.6*	0.0	0.0	0.0	--
7	0	3	331	178	53.7	3.3	6.1	0.0	1.3
	5	3	433	262	60.5	2.3	3.6	0.8	1.1
	10	3	843	126	14.9*	7.9	8.9	5.6	2.5*
6 & 8	0	4	309	173	56.0	1.8	3.3	0.0	1.1
	10	4	868	110	12.6*	9.1**	9.9	6.9	2.8*
9	10	6	444	55	13.3*	23.6*	30.9	0.0	3.2**
	10	8	400	129	32.3*	24.0*	24.2	20.2	24.8*
7	0	9	211	155	73.4	2.0	2.3	1.3	1.3
6	5	9	137	39	28.4*	17.9*	20.0	0.0	8.7*
7 & 9	10	9	238	56	19.3*	30.4*	32.1	0.0	17.7*

† Number of larvae transferred after treatment

‡ $100 \times \frac{\text{No. abnormal}}{\text{Total eclosed of appropriate sex}}$

* Significant at the 1% level

** Significant at the 5% level

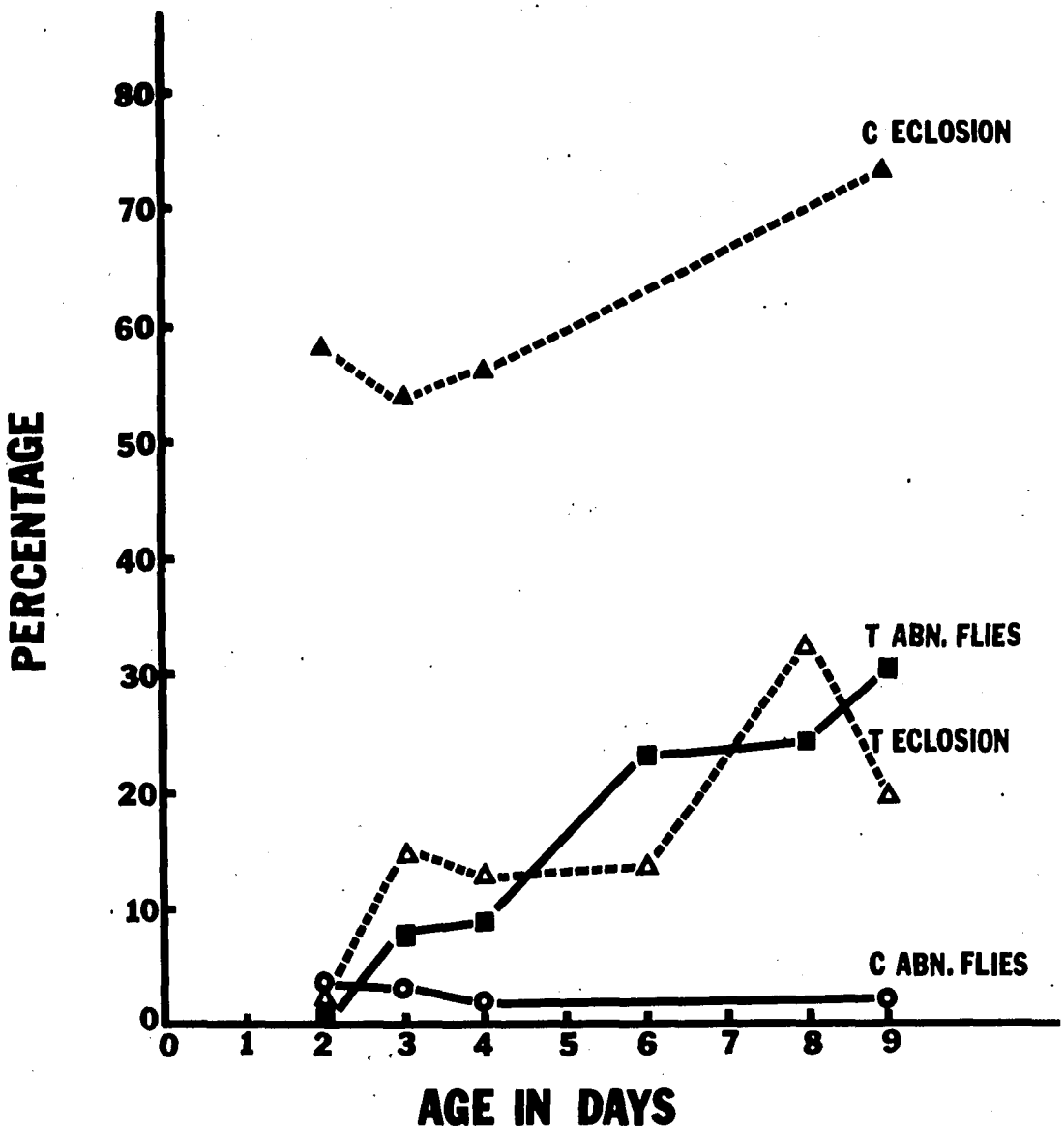

Figure 3. - Effect on phenocopy induction and viability of treating stock 2 larvae with 10 µg/ml actinomycin D. T = Treated series. C = Control series. Data from Table 3.

frequency of abnormal flies when 9-day old larvae are treated.

A significant difference was found between the wild-type and the multiple mutant stock in the effect that actinomycin D produces on the adult sex ratio (♀♀/♂♂); this effect is discussed in detail elsewhere (Margulies, submitted for publication). Since the mutant stock shows a steadily increasing adult sex ratio from larvae treated on successive days in the 3-9 day period, most abnormal flies are females. It should be noted that after treatment of 6-day old larvae with 10 µg/ml actinomycin D none of the 13 surviving males and 13 of 42 surviving females were abnormal. This difference in phenocopy production between males and females approaches significance; if real it may be due to males having a greater sensitivity to the lethal than to the phenocopy effect of the antibiotic as compared to females. This explanation is supported by the observation that many treated males die in the larval stage, regardless of how close to pupation they are treated.

Specific phenocopy effects of actinomycin D: The results for the specific types of phenocopies induced in the wild-type stock by 40 µg/ml actinomycin D at different stages of development are summarized in Table 4. The phenocopies obtained were variable in expressivity and were often asymmetrical. Some flies showed more than one phenocopy; some phenocopies tended to appear together. These concurrences included, for example, rough eye and aristaless as well as eyeless and double antenna or arista. Very rare phenocopies (Bar-like eye; malformed eye, head or proboscis; bubbled wing) as well as abnormalities which seem to be just as frequent in control flies (abdominal defects; missing or

TABLE 4

Phenocopies induced by actinomycin D treatment (40 µg/ml) of wild-type larvae of different ages. Data obtained from Experiments 1 and 2 (Table 2). Percentages of phenocopies given in terms of total number of flies eclosed

Phenotypes Observed	Age in days					
	Combined Controls	Treated				
		Experiment 1		Experiment 2		
	5-10	5	6	6	8	10
Eye, rough	0	1.3	0	2.1	44.9	57.4
Aristaless	0	0	0	0	2.3	7.7
Genitalia, missing or abnormal	0.3	2.6	5.4	3.7	1.9	0
Halteres, missing	0.1	3.8	4.3	3.4	1.4	1.9
Thorax, bithorax or other abnormalities	0	1.3	2.2	1.5	3.1	1.3

Aristaless	0	0	0	0	2.3	7.7
Genitalia, missing or abnormal	0.3	2.6	5.4	3.7	1.9	0
Halteres, missing	0.1	3.8	4.3	3.4	1.4	1.9
Thorax, bithorax or other abnormalities	0	1.3	2.2	1.5	3.1	1.3
Legs, missing	0.2	2.6	3.2	8.9	9.2	7.7
Legs, malformed or crippled	0.1	2.6	2.2	1.5	3.1	3.9
Eye, erupt-bulge	0	0	0	0	2.7	3.9
Eyeless	0	1.3	1.1	0.3	1.6	0
Antenna & arista, double	0	0	0	0	0.8	0
Antennaless	0	0	0.3	0.2	0.6	0
Palp, double, missing or malformed	0	2.6	1.1	0	0.6	0.6
Total flies eclosed	911	78	93	327	512	155

abnormal bristle) are not included in Table 4. Phenocopies, such as grossly malformed heads with one or both eyes missing, were seen in fully formed, dissected pupae; these malformations probably prevented eclosion. Dissected pupae or partially eclosed flies are, however, not included in the results presented here.

The controls showed few phenocopies and no pattern among these; accordingly, all controls are combined in Table 4. In the experimentals the pattern of phenocopies can be analyzed only within the same experiment; that is, where samples of larvae treated at different ages are taken from one growing population. In Experiment 1, no detectable difference occurred between the frequency with which any specific phenocopy appeared after treatment on days 5 and 6. In Experiment 2 the last four types of phenocopies listed in Table 4 occurred in frequencies too low for meaningful comparison. The frequencies of the other phenocopies in Experiment 2 (Table 4 and Figure 4) increase, decrease or remain the same in the 6-8-10 day age period. For example, rough eye -- the most frequently occurring phenocopy -- increases significantly when 8-day old larvae are treated as compared to day 6, but does not increase significantly between day 8 and day 10. A similar pattern is found for erupt-bulge. (The erupt-bulge phenocopy includes erupt eyes, bulged eyes and combinations of both). Aristaless is significantly increased in 10-day old larvae as compared with either day 6 or day 8. On the other hand, a comparison of days 6 and 10 shows a significant decrease in genital phenocopies. Thorax, haltere, and leg phenocopies are found in similar frequencies on all three days.

The significance of differences in phenocopy frequencies listed in

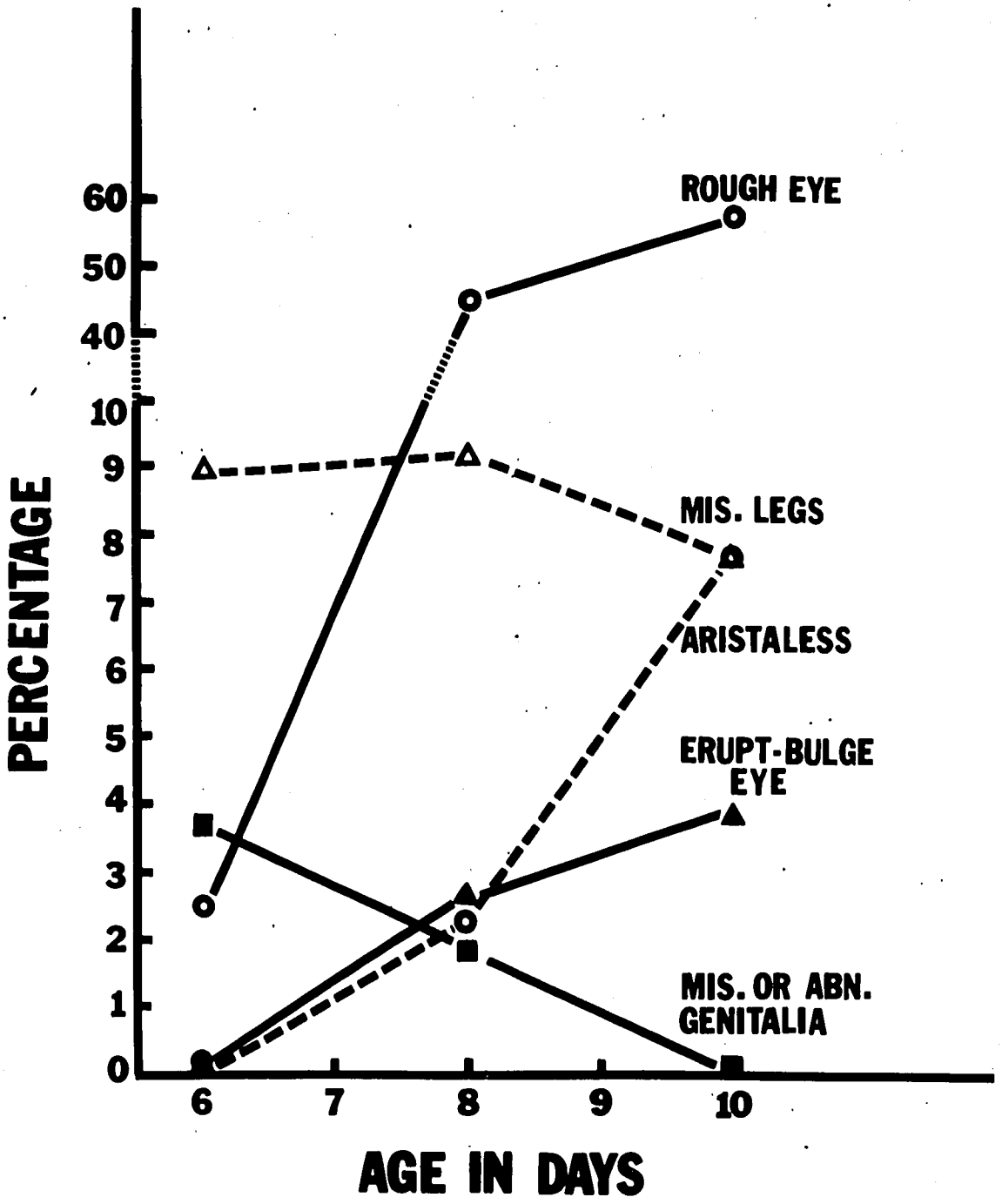

Figure 4. - Frequencies of different phenocopies among eclosed flies after treatment of wild-type larvae of different ages with 40 ug/ml actinomycin D. Mis. = Missing. Abn. = Abnormal. Data from Table 4.

the preceding paragraph was usually based on obtaining values of P .05, both when the total number of eclosed flies and the total number of abnormal flies were used as the total fly population. Erupt-bulge was the only exception to this double test of significance; in this case only the test using the number of eclosed flies was significant in comparing days 6 and 8. The number of eclosed flies is really the better criterion, since the frequency of abnormal flies goes up greatly when 8-day and 10-day old larvae are treated due, mainly, to the high frequency of rough eye. Although the percentages of flies with different kinds of phenocopies in Experiments 3 and 4 are not shown, the treatment of 6-day old larvae with 30 µg/ml produced similar results to those in Experiments 1 and 2 for this age. Moreover, other experiments, not reported here, involving treatment of 8-day and 10-day old larvae with 50 µg/ml (where pupation starting time differed only by about a half day from that in Experiment 2) confirmed the pattern of all the phenocopies described here for days 8 and 10.

No difference was usually found in the frequencies with which different phenocopies occurred among wild-type males and females. The exceptions include a significantly higher incidence of genital phenocopies among males for 6-day old larvae in Experiment 2, and a higher frequency of missing halteres among females for 8-day old larvae in the same experiment. Since the latter finding also applies after treatment of 6-day old larvae with 30 µg/ml and 5-day old larvae with 20 and 40 µg/ml (when the results of the last two concentrations are combined), it may represent a real difference.

The pattern of the main types of phenocopies in the mutant stock (stock 2) can be seen in Table 5 and Figure 5. During the 6-8-9 day age

TABLE 5

Phenocopies induced by actinomycin D treatment (10 µg/ml) of stock 2 larvae of different ages. Data obtained from Experiments 6-9 (Table 3). Percentages of phenocopies given in terms of total number of flies eclosed

Phenotypes Observed	Ages in days					
	Combined Controls	Treated				
	3-9	3	4	6	8	9
Legs, missing	0	0	0	7.3	6.2	3.6
Legs, malformed or crippled	2.0	4.0	0.9	10.9	12.4	12.5
Eye, erupt-bulge	0	0	0	1.8	6.2	17.9
Eyeless	0	0	0.9	1.8	1.6	0
Antenna & arista, double	0	0	0	1.8	1.6	3.6

	Combined Controls	Treated				
	3-9	3	4	6	8	9
Legs, missing	0	0	0	7.3	6.2	3.6
Legs, malformed or crippled	2.0	4.0	0.9	10.9	12.4	12.5
Eye, erupt-bulge	0	0	0	1.8	6.2	17.9
Eyeless	0	0	0.9	1.8	1.6	0
Antenna & arista, double or triple	0	0	0	1.8	1.6	3.6
Wing, cut	0	0.8	2.7	0	0	1.8
Total flies eclosed	506	126	110	55	129	56

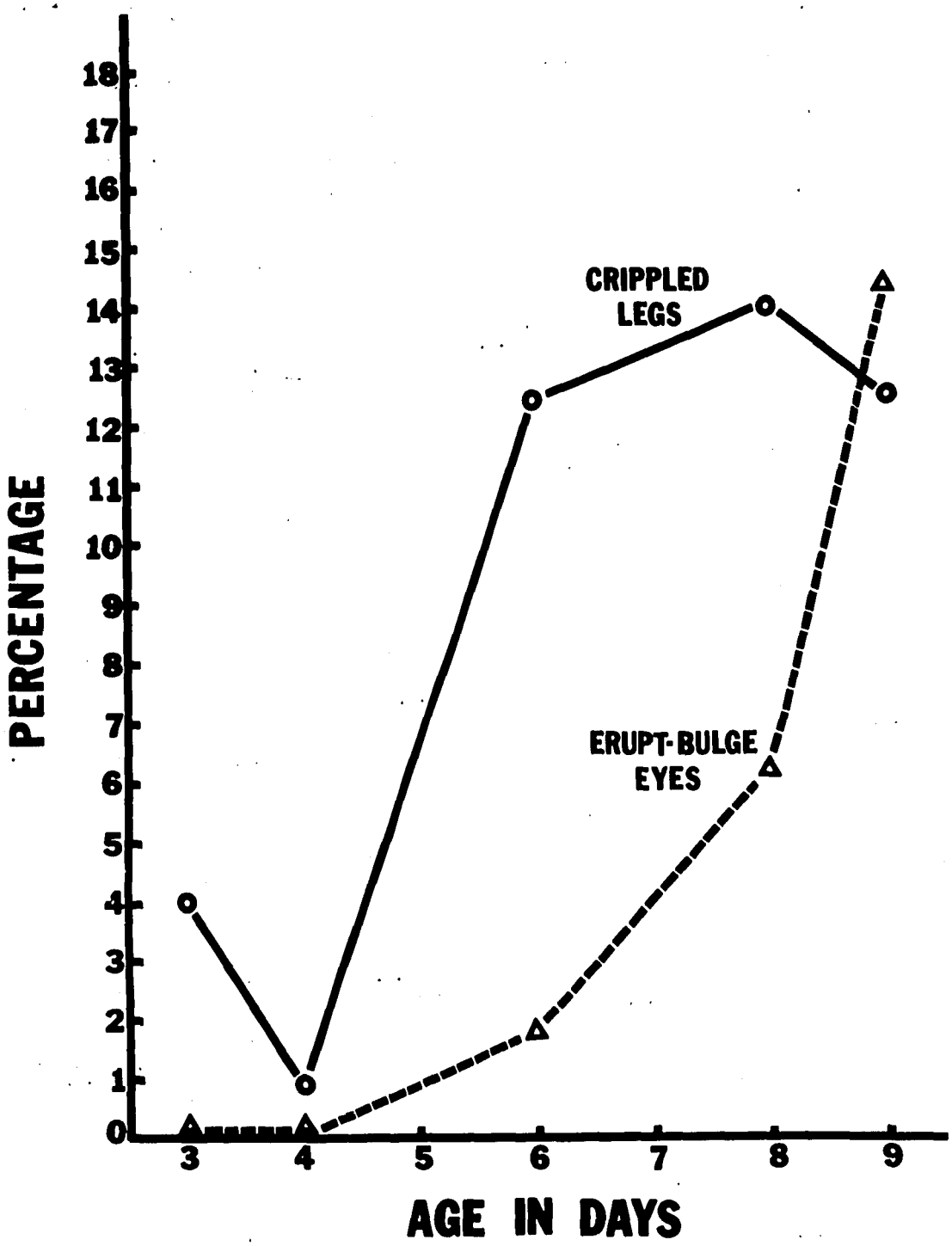

Figure 5. - Frequencies of different phenocopies among eclosed flies after treatment of stock 2 larvae of different ages with 10 ug/ml actinomycin D. Data from Table 5.

period, a pattern similar to that found in the wild-type stock occurs with respect to erupt-bulge and leg phenocopies (all leg abnormalities were combined in Figure 5 under one type, crippled); that is the frequency of erupt-bulge increases between day 6 and day 9, whereas crippled does not change significantly. Rough eye could not be scored in this stock, since Minute females normally tend to have this phenotype. Aristaless, and phenocopies affecting genitalia, halteres and thorax induced in the wild-type stock did not occur in the mutant stock. To test whether the absence of these phenocopies was attributable to the lower concentrations used in the case of the mutant stock, or to a stock-dependent response, larvae of stock 2 were treated with 40 µg/ml actinomycin D. Although very few flies eclosed, haltere and thorax phenocopies were found, indicating that the absence of certain phenocopies after 10 µg/ml treatment is attributable to low concentration.

Comparison of frequencies of specific phenocopies in different stocks: Are certain types of phenocopies induced with actinomycin D in greater frequencies in certain genotypes? Such a dependence was demonstrated for other phenocopying agents in Drosophila (Goldschmidt and Piternick 1957; Herskowitz and Norton 1963). The frequency of erupt-bulge in stock 2 on days 8 and 9 is significantly higher ($P < .01$) than in the wild-type stock on days 8 and 10. Another indication that certain genotypes are more easily phenocopied with respect to certain traits was obtained in another experiment in which 8-day old larvae of stock 3 (M/Basc ♀♀ × Basc/Y ♂♂) were treated with 20 µg/ml actinomycin D. A relatively high frequency of double or triple antenna and arista was found in the non-Minute progeny — 4.8 percent for Basc/Y plus Basc/Basc

individuals. The 0.7 percent frequency for the M/Basc progeny was significantly lower ($P<.05$). Although the lower frequency may be correlated with the slower development of M/Basc as compared with non-Minute larvae, this is probably not the explanation for the significant difference. For the 4.8 percent antenna-arista phenocopy frequency in Basc/Y and Basc/Basc individuals treated with 20 µg/ml is significantly higher ($P<.01$) than that for wild-type flies treated at different larval ages (4, 5, 6, 8, and 10 days) with concentrations of 20-40 µg/ml, and is significantly higher ($P<.05$) in six out of seven experiments in which wild-type larvae 8-10 days in age were treated with 50 µg/ml. Since the time of pupation is similar for stock 3 and wild-type, the difference is probably best explained as being due to a difference in phenocopying susceptibility of different genotypes at the same developmental stage.

Effect of actinomycin D treatment on the frequency of X-linked recessive lethals: It is possible that the "phenocopies" reported here are the result of actinomycin D-induced somatic mutations. Assuming that germ and somatic cells would be affected in the same way by actinomycin D, the frequency of X-linked recessive lethals was determined in the gametes of treated males. Although Burdette (1961) and Mukherjee (1965) demonstrated that actinomycin D has no detectable effect on the spontaneous frequency of X-linked recessive lethals in sperm, it was necessary to test this finding under the present experimental conditions, which differ greatly from theirs. Wild-type males treated as 6-day old and 8-day old larvae with 40 µg/ml actinomycin D and control males (Experiment 2) eclosing over a period of 5 days were mated on the day of

eclosion to Basc virgin females (1 ♀:1 ♂). Using the Basc technique, no difference was detected in mutant frequency between the experimental (3 lethals/1220 tested sperm; 0.24%) and control flies (3/920; 0.33%). Since the somatic mutation explanation is not supported by this germ-line mutation study, it is concluded that actinomycin D is producing a phenocopy rather than a mutational effect.

Effect of different doses of the nucleolus organizer region on phenocopy frequency: Assuming that the induced phenocopies are a consequence of the inhibition of DNA-dependent RNA synthesis by actinomycin D, the possibility exists that they result from a preferential inhibition of ribosomal RNA synthesis. Such a differential effect of actinomycin D on the synthesis of the RNA class has been indicated in work with other organisms (Perry 1962; Georgiev 1963). An experiment was performed, therefore, to test the frequency of actinomycin D-induced phenocopies in females with three nucleolus organizer regions as compared to males with one such region, since it has been demonstrated by Ritossa and Spiegelman (1965) that DNA of the nucleolus organizer region codes for rRNA in Drosophila melanogaster. On the assumption that the concentration of actinomycin D is relatively low and, therefore, limiting in the nucleus, there should be a greater chance of inhibiting one nucleolus organizer region than three such regions.

Cross 4 (+/+ ♀♀ × w.Y^{S}/Y^{Lc} ♂♂) generates +/w.Y^{S} ♀♀ (wild-type females with 3 nucleolus organizers) and +/Y^{Lc} ♂♂ (wild-type males with one nucleolus organizer). The expectation which is thus being tested is that a greater frequency of phenocopies should be induced in the male (having fewer nucleolus organizers) than in the female, if pheno-

copies result mainly from a selective inhibition of rRNA synthesis. Treatment of 8-day old larvae with 50 µg/ml actinomycin D produced no detectable difference in the percentage of phenocopies among males and females (although females were more sensitive to the killing effect of actinomycin D) (Table 6). Since pupation in this experiment started 8 days after hatching, i.e., 1-2 days earlier than in the inbred wild-type stock (perhaps due to heterosis), the stage of development of these 8-day old larvae probably corresponds to that of 10-day old wild-type larvae in Experiment 2. The types and frequencies of phenocopies obtained confirm this. The finding that over 50 percent of the treated larvae eclosed as adults indicates that the concentration of actinomycin D used is relatively low. The results of this experiment suggest that the actinomycin D-induced phenocopies of adult traits are not due mainly to a preferential inhibition of transcription to rRNA.

TABLE 6

Effect of actinomycin D (50 µg/ml) on ($+/w.Y^{S}$) females with three nucleolus organizers and ($+/Y^{Lc}$) males with one, treated as 8-day old larvae

	No. Larvae	No. Eclosing $+/w.Y^{S}$ ♀♀	No. Eclosing $+/Y^{Lc}$ ♂♂	% Eclosion	% Abnormal Adults †	% Abnormal ♀♀ †	% Abnormal ♂♂ †	Adult Sex Ratio ♀♀/♂♂
Control	497	224	241	93.6	3.2	0.4	5.8	0.93
Treated	744	167	248	55.8*	69.4*	73.6	66.5	0.67**

† 100 x $\frac{\text{No. abnormal}}{\text{Total eclosed of appropriate sex}}$

* Significant at the 1% level

** Significant at the 5% level

GENERAL DISCUSSION

The importance of using axenic conditions for Drosophila research cannot be overestimated. Sang and McDonald (1954) found many complications due to microbial, especially yeast symbionts, of Drosophila when they tried to reproduce the results of Rappoport on the induction of phenocopies with chemical agents. It is equally desirable to grow Drosophila melanogaster in a chemically-defined medium in order to reduce the variability which might result from known or unknown constituents of the standard culture medium. In the work presented here, it was important to have a medium free of DNA to prevent the binding of actinomycin D to this nucleic acid. Another advantage of using germ-free, chemically-defined conditions for larval growth is that larval development is slower. This provides a longer period of experimentation -- making the treatment of different stages more easily distinguishable.

Two main types of results were obtained which indicate that actinomycin D does not persist in the larval cells for many days after larvae are transferred to an actinomycin D-free medium: 1) phenocopies absent when younger larvae were treated occurred after treatment of older larvae; 2) survival increased then decreased with increasing age of treatment. This reversibility agrees with the results of Clever and Lombard (1966) who have demonstrated the reversibility of actinomycin D-inhibition of RNA synthesis in Chironomus larvae.

Although the percentage of eclosed flies with phenocopies was high (ranging from 17 to 72 percent of adults eclosing from 6 and 10 day-old wild-type larvae treated with 40 µg/ml actinomycin D), with the ex-

ception of rough eye the frequencies of specific types of phenocopies were relatively low. Thus, there seems to be a correlation between the number of known loci affecting the development of an organ in a particular way (Bridges 1944) and the occurrence of actinomycin D-induced phenocopies of the particular type. For example, rough eye, which can be produced by 26 loci, can be induced by actinomycin D in more than 50 percent of the eclosed flies, whereas eyeless and aristaless, each of which can be produced by 2-3 loci, occur in about 2-7 percent of treated flies.

A normal or nearly normal phenotype is usually obtained in Drosophila when the equivalent of one member of a gene pair is operative -- that is, most Drosophila mutants are recessive. It is expected, therefore, if actinomycin D acts at the transcription level, that phenocopies usually result only when both members of a gene pair are affected simultaneously. In the case of rough eye, however, some phenocopies might be produced by affecting two or more single members of the numerous pairs that can affect eye texture.

After treatment of wild-type larvae 1 and 3 days in age and mutant larvae 1-3 days in age with actinomycin D, the percentage of abnormal adults was not significantly higher than in the control. Since two separable effects of actinomycin D have been observed, larval death (which may be thought of as the phenocopy of the effect of larval-lethal genes) and induction of phenocopies of adult traits, the sensitivity of larvae to both effects must be considered in the conclusions drawn. When somewhat older larvae (4-6 day old wild-type, and 4 and 6-day old mutant larvae) are treated, phenocopies of adult traits appear in a significant percentage of the flies that eclose, even though substantial killing occurs in the larval stage. It is reasonable to assume, there-

fore, even though lethality was high after treatment of younger larvae, that a significant number of the adult survivors should have had phenocopies had such changes been induced. The results suggest, therefore, that these phenotypic changes are not inducible in the early stages (1-3 days) of larval development. (The results of one experiment, treatment of 2-day old wild-type larvae with 30 µg/ml actinomycin D -- seemingly contrary to this conclusion -- are not evaluated because they do not meet the criterion for minimum eclosion percentage. This result can be explained, however, in two ways consistant with the hypothesis under discussion. First, this in the only one of 9 experiments done with young larvae of stocks 1 and 2, 1-3 days in age, that gave a significant frequency of phenocopies. Second, although actinomycin D ordinarily does not persist in larval tissues, it may have persisted in this case and produced phenocopies at a later, phenocopy-inducible stage of development. All three atypically abnormal flies in this experiment had genital phenocopies and might have been produced if actinomycin D persisted to the 5 or 6-day old stage, the time of highest induction of genital abnormalities).

The results presented here demonstrate a differential phenocopy effect at different stages of development when larvae of wild-type or mutant stocks are treated with actinomycin D. The differential production of phenocopies of adult traits is indicated, both by the absence of phenocopies when young larvae, 1-3 days in age, are treated, and by the different pattern of phenocopies whose frequencies increase, decrease, or remain the same when larvae of different, older, ages are treated. The changes in sensitivity of larvae of different ages to the separable,

lethal effect of actinomycin D indicate, indirectly, differential phenocopying of the effect of larval-lethal genes.

The possibility of a differential permeability of the imaginal discs to actinomycin D at different developmental stages must be considered, since this might be the cause of the observed differential phenocopy effect. This explanation is unlikely because different phenotypes of the same organ can be effected at different developmental stages. For example, the phenocopy eyeless occurs in similar, low frequencies on days 5-8, whereas rough and erupt-bulge eye are found mainly on days 8 and 10 (Table 4). The phenocopy double antenna-arista and antennaless occurs in low frequencies when 6-10-day old larvae are treated (the former phenocopy was induced in Experiments 3 and 4, Table 2; Experiment B, Table 1, and in other experiments not reported here); aristaless, however, has not been induced in 6-day old larvae in any of the experiments, although it is consistently found after treatment of 8 and 10-day old larvae. Moreover, Perez-Davila and Baker (1967) have reported that actinomycin D treatment of second instar larvae results in the inhibition of mitosis in the eye-antenna imaginal disc. Under our experimental conditions the second instar probably includes days 3-4, a developmental period during which actinomycin D has either no detectable effect or induces few phenocopies respectively.

In addition to the inhibition of DNA-dependent RNA synthesis, other possible effects of actinomycin D must also be considered. It has been reported that relatively high concentrations of actinomycin D induce chromosome breakage in locust spermatocytes (Jain and Singh 1967). It is unlikely that the phenocopies induced by actinomycin D in the present

work are due to a mutagenic effect of the antibiotic, since the relatively low concentrations used to induce phenocopies had no effect on the frequency of X-linked recessive lethals. Burdette (1961) and Mukherjee (1965) had earlier failed to detect an increase in X-linked recessive lethals in Drosophila exposed to actinomycin D.

The inhibition of energy metabolism by actinomycin D (an effect unrelated to that on RNA synthesis) can also, indirectly impair protein synthesis (Revel and Hiatt 1964; Laszlo et al. 1966). Since the effect on energy metabolism was prevented by glucose (Honig and Rabinowitz 1965), it is possible that the sucrose in the chemically-defined medium used in the present work might act in a similar fashion.

That the mode of action of actinomycin D may be complex and that experiments using this antibiotic as a tool should be interpreted with caution, is also indicated by the findings that 1) actinomycin D exposure causes degradation of RNA synthesized prior to exposure in mouse fibroblasts (Wiesner et al. 1965); 2) the synthesis of certain proteins can be stimulated by actinomycin D (Papaconstantinou et al. 1966); and 3) actinomycin D can inhibit the synthesis, as well as the degradation, of an enzyme (Kenney 1967).

Although the effect of actinomycin D on RNA and protein synthesis has been studied in the larval salivary glands of Drosophila busckii (Ritossa and Pulitzer 1963), the effect of the antibiotic on macromolecular synthesis in the imaginal discs of Drosophila melanogaster larvae has not been investigated. Thus a causal relationship between actinomycin D-induced phenocopies of adult traits and the effect of actinomycin D on nucleic acid and protein synthesis can, at present, only be inferred.

If the actinomycin D-induced phenocopies are a consequence of the inhibition of RNA synthesis, the differential phenocopy effects provide evidence for differential transcription of the genome during the development of Drosophila melanogaster. The results indicate that: 1) genes which affect the differentiation of imaginal discs are activated in the third larval instar (days 5-10); 2) a different developmental timing of transcription occurs in different imaginal discs; for example, genital disc primarily on days 5-8 and eye disc on days 5-10; 3) differential transcription occurs within the differentiating imaginal disc as seen by the different eye and antenna-arista phenocopies induced at different larval stages.

If, on the other hand, the phenocopies are due mainly to the inhibition of protein synthesis, the differential phenocopy induction may indicate a differential translation of messenger transcripts which had been synthesized early in development, i.e., the egg stage or very early larval stages. The latter possibility has to be considered in view of the evidence that RNA templates synthesized at one stage of embryonic development of the sea urchin are stored and translated in a later stage (Infante and Nemer 1967).

The experiments presented here can not distinguish between these two alternatives. Evidence from the work of other investigators, however, suggests that the phenocopies induced by actinomycin D in the present work may be due, primarily, to the inhibition of RNA synthesis, since: 1) concentrations of actinomycin D which inhibit RNA synthesis almost completely, but have little effect on protein synthesis produce gross abnormalities in sea urchin embryos (Gross and Cousineau 1963; 1964); concentrations of actinomycin D which reduce RNA synthesis by

90 percent in rat liver do not affect protein synthesis (Revel and Hiatt 1964); in Drosophila busckii, RNA synthesis was completely blocked in the larval salivary glands, but protein synthesis was not detectably affected by actinomycin D (Ritossa and Pulitzer 1963); in the snail, Ilyanassa, a concentration of actinomycin D which represses RNA synthesis by 72 percent, and protein synthesis only by 24 percent affects the differentiation of specific organs during different embryonic stages (Collier 1966); 2) the concentrations of actinomycin D which induce phenocopies in the present experiments are relatively close to the threshold level, since reducing the concentration to one half (10 ~ug/ml) in wild-type produces no detectable adult phenocopy effect; 3) using four different kinds of inhibitors of protein synthesis, Marzluf (1967) found that three of the inhibitors produced no phenocopies in Drosophila melanogaster and one inhibitor had a limited effect in one strain only.

The alternative is favored, therefore, that the actinomycin D-induced phenocopies result from the inhibition of DNA-dependent RNA synthesis. If this alternative is valid, the differential production of phenocopies provides supporting evidence for the hypothesis that differential gene action occurs during the differentiation of imaginal buds in Drosophila melanogaster.

Although the present results with a variable number of nucleolus organizer regions suggest that phenocopies are not due to a selective inhibition of rRNA synthesis by actinomycin D, they clearly do not reveal the relative role or extent of the inhibition of synthesis of the various classes of RNA.

SUMMARY

Drosophila melanogaster larvae of a wild-type and several mutant stocks, cultured in an exenic chemically-defined medium, were treated for one day with different concentrations of actinomycin D at different stages of development. Phenocopies affecting various organs of the adult occurred in different frequencies and in different patterns depending on the age of treatment. Assuming that the phenocopies were due primarily to the inhibition of DNA-dpendent RNA synthesis by actinomycin D, the differential phenocopy effect indicates that: 1) genes which affect the differentiation of imaginal discs are activated in the third larval instar. 2) transcription of these genes in different imaginal discs occurs at different times in the third instar. 3) differential transcription of the genome occurs within an imaginal disc; i.e., genes having different phenotypic effects on an organ are activated at different times.

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PART II

THE GENETIC BASIS OF ACTINOMYCIN D-INDUCED PREFERENTIAL KILLING OF *DROSOPHILA MELANOGASTER* MALES

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THE GENETIC BASIS OF ACTINOMYCIN D-INDUCED PREFERENTIAL KILLING OF *DROSOPHILA MELANOGASTER* MALES*

INTRODUCTION

In the course of investigating actinomycin D-induced phenocopies in *Drosophila melanogaster* (Margulies, submitted for publication), males of a particular genotype were found to be killed preferentially by actinomycin D. The high sensitivity of these males to the antibiotic increased with larval age and resulted in an adult sex ratio (♀♀/♂♂) as high as 25:1 when older larvae were treated.

The present study was designed to elucidate the relationship between the preferential killing effect of actinomycin D and the genotype of the "sensitive" male. It will be demonstrated that this selective detrimental effect of actinomycin D is dependent on the presence in the male of an X-limited gene which is hypothesized to inhibit the action of other X-limited genes.

MATERIALS AND METHODS

The following stocks and genotypes were used: (1) +/+ ♀♀ × +/Y ♂♂ (Oregon-R, wild-type stock). (2) $\underline{M/sc^{S1}\ bb^{1}\ B\ In49\ oc\ ptg\ sc^{8}}$ ♀♀ × $\underline{sc^{S1}\ bb^{1}\ B\ In49\ oc\ ptg\ sc^{8}}$/Y ♂♂. This stock is henceforth referred to as

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M/bb^1 B oc ♀♀ × bb^1 B oc/Y ♂♂. M = Minute bristles; sc^{S1} sc^8 = scute inversion; bb^1 = bobbed bristles, lethal; B = Bar eye; In49 = inversion 49; oc = ocelliless; ptg = pentagon. (3) M/sc^{S1} B InS w^a sc^8 ♀♀ × sc^{S1} B InS w^a sc^8/Y ♂♂, henceforth referred to as M/Basc ♀♀ × Basc/Y ♂♂. w^a = apricot eye color; InS = inversion S. (4) +/oc ptg ♀♀ × oc ptg/Y ♂♂, henceforth referred to as +/oc ♀♀ × oc/Y ♂♂. (5) Y/y f :═ ♀♀ × sc^{S1} bb^+ B In49 oc ptg sc^8/Y ♂♂. The males are henceforth referred to as bb^+ B oc/Y. y = yellow body color; f = forked bristles; :═ = attached X's; bb^+ = wild-type bobbed locus. (6) Y/y w f :═ ♀♀ × In49 Fl v g/Y ♂♂. w = white eye color; Fl = Female lethal; v = vermillion eye color; g = garnet eye color. (7) Y/y f :═ ♀♀ × bb^1 B oc/Y (stock 2) ♂♂. (8) Y/y f :═ ♀♀ × y sc^{S1} B In49 ct^{ns} sc^8/Y ♂♂. The male is henceforth referred to as y B ct^{ns}/Y. ct^{ns} = cut wings. (9) y v bb .═ ♀♀ × Y^S·X InEN B f v y·Y^L y^+ ♂♂. .═ = attached X's; Y^S = shorter arm of Y chromosome, here attached to the X; InEN = inversion entire; Y^L = longer arm of Y, here with y^+ marker and attached to the X chromosome.

Eggs laid in a 2-hour period were surface sterilized using Geer's method (Geer 1963) and distributed axenically into petri dishes containing sterile, chemically-defined Geer's medium (Geer 1965). Larvae of a given age (stated as the number of days after hatching) were transferred axenically in known numbers to new petri dishes containing actinomycin D - Geer's medium on which they were fed for about one day (usually 22-23 hours). (The actinomycin D was supplied through the courtesy of Merck, Sharp and Dohme). After this treatment, dead larvae were counted and surviving larvae were transferred to sterile, standard Drosophila medium. This method of treatment was used in experiments presented in Tables 1 and

3-8. (Since some larvae crawled out of the actinomycin D-medium, the larval population on which the percentage of eclosion is based is the number of dead larvae plus the number transferred after treatment).

The method of treatment used in the experiments recorded in Table 2 differed in that actinomycin D solution was pipetted directly into the petri dishes in which larvae were developing and the duration of treatment was usually 18-21 hours. (The larval population on which the percentage of eclosion is based in these experiments is the number of surviving larvae transferred after treatment). All cultures were kept at $24^{\circ} \pm 0.5^{\circ}$C, and only those were used which remained axenic until the first adults were examined under narcosis.

RESULTS AND DISCUSSION OF SPECIFIC EXPERIMENTS

Effect of actinomycin D on the sex ratio of adults of different genotypes: Table 1 and Figure 1 summarize the effect of different concentrations of actinomycin D on the adult sex ratio ($\varphi\varphi/\sigma\sigma$) of the wild-type stock after treatment of larvae of different ages. Only once did a significant difference occur between the experimental and control sex ratio -- after 4-day old larvae were treated with 30 μg/ml. No significant increase in the sex ratio was found in the wild-type stock in 8 additional experiments (not reported here) which treated 6-10 day old larvae.

The effect of actinomycin D on the adult sex ratio of stock 2 is shown in Table 2 and Figure 2. No difference is detected between control and experimental flies after 1 and 2-day old larvae are treated with 5 μg/ml actinomycin D. The sex ratio increases significantly after treatment of 3-day old larvae with 10 μg/ml; remains at about this level on

TABLE 1

Effect of actinomycin D on the adult sex ratio (♀♀/♂♂) after treating wild-type larvae of different ages. 0 = Control

Act. D µg/ml	Age of Larvae (days)	No. Larvae	No. Eclosing ♀♀	No. Eclosing ♂♂	% Eclosing	% Abnormal Adults	Adult Sex Ratio (♀♀/♂♂)
0	1	478	214	218	90.4	3.0	0.98
20	1	1400	137	164	21.5**	3.3	0.84
0	3	166	80	76	94.0	1.3	1.05
20	3	879	359	310	76.1**	3.3	1:2
40	3	678	4	7	1.6**	9.1	--
0	4	315	120	145	84.1	3.0	0.83
30	4	1800	201	80	15.6**	7.8*	2.5**
0	5	273	118	123	88.3	1.2	0.96
20	5	588	85	90	29.8**	5.7*	0.94
40	5	1025	31	47	7.6**	17.9**	0.66
0	6	234	103	105	88.8	10.1	0.98

0	5	273	118	123	88.3	1.2	0.96
20	5	588	85	90	29.8**	5.7*	0.94
40	5	1025	31	47	7.6**	17.9**	0.66
0	6	234	103	105	88.8	10.1	0.98
30	6	1087	238	161	36.7**	19.3**	1.48
0	6	271	128	116	90.0	2.1	1.1
20	6	560	133	144	49.5**	7.6**	0.92
40	6	833	49	44	11.2**	17.2**	1.1
0	6	136	54	69	90.4	1.6	0.78
40	6	1300	164	163	25.2**	17.1**	1.0
0	8	223	113	93	92.4	1.9	1.2
40	8	1178	270	242	43.5**	62.3**	1.1
0	10	114	46	51	85.1	4.1	0.9
40	10	593	87	68	26.1**	72.9**	1.3

* Significant at the 5% level

** Significant at the 1% level

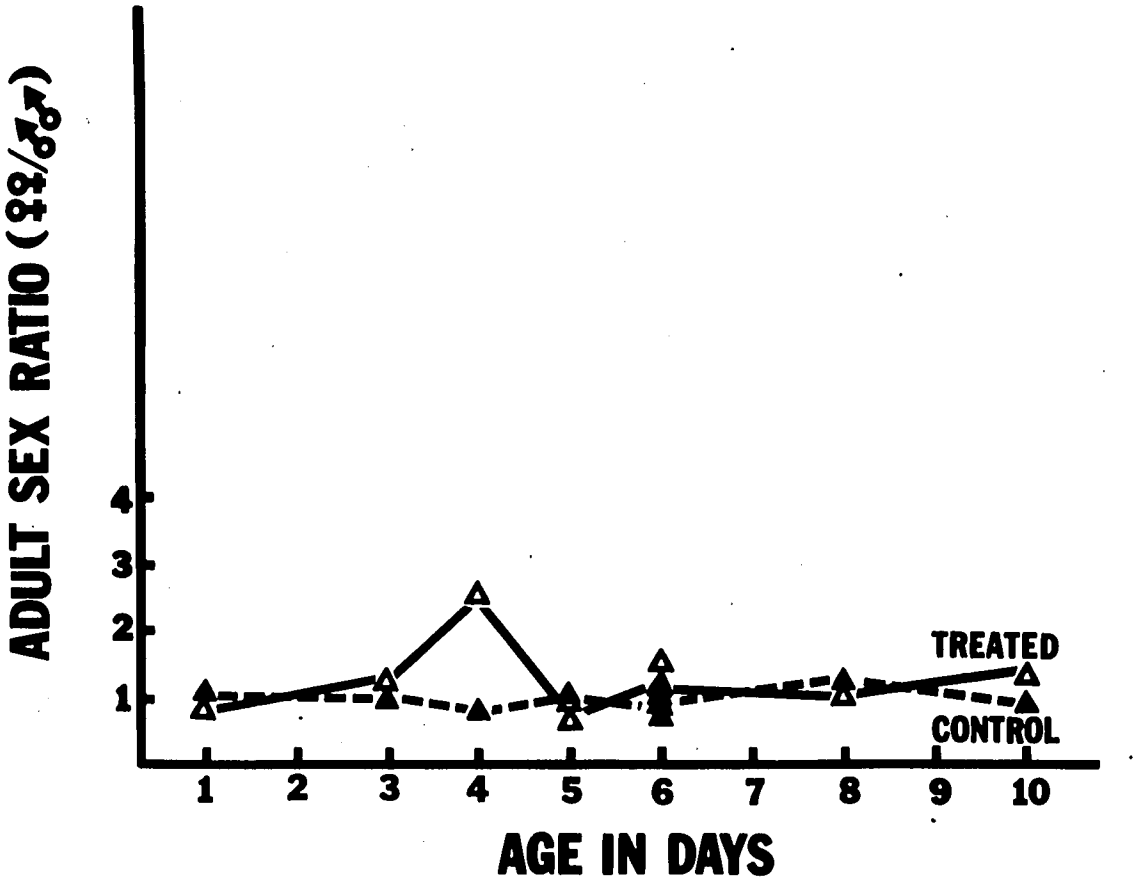


Figure 1. - Effect of actinomycin D on the adult sex ratio (♀♀/♂♂) after treating wild-type larvae of different ages with different concentrations (20-40 µg/ml). Data taken from Table 1. When more than one value occurs for a given day, the line is drawn through their average.

TABLE 2

Effect of actinomycin D on the adult sex ratio (♀♀/♂♂) after treating stock 2 larvae of different ages. Cross: M/bb^1 B oc × bb^1 B oc/Y. 0 = Control

Act. D µg/ml	Age of Larvae (days)	No. † Larvae	No. Eclosing M/bb^1 B oc ♀♀	No. Eclosing bb^1 B oc/Y ♂♂	% Eclosing	% Abnormal Adults	Adult Sex Ratio (♀♀/♂♂)
0	1	162	61	40	62.3	3.0	1.5
5	1	308	39	31	22.7**	2.8	1.3
10	1	468	2	0	0.4**	0.0	--
0	2	350	120	83	58.0	3.4	1.4
5	2	665	101	75	26.8**	4.5	1.3
10	2	418	6	5	2.6**	0.0	--
0	3	331	99	79	53.7	3.3	1.3
5	3	433	140	122	60.5	2.3	1.1
10	3	843	90	36	14.9**	7.9	2.5**
0	4	309	91	82	56.0	1.8	1.1
10	4	868	81	29	12.6**	9.1*	2.8**

5	2	665	101	75	26.8**	4.5	1.5
10	2	418	6	5	2.6**	0.0	--
0	3	331	99	79	53.7	3.3	1.3
5	3	433	140	122	60.5	2.3	1.1
10	3	843	90	36	14.9**	7.9	2.5**
0	4	309	91	82	56.0	1.8	1.1
10	4	868	81	29	12.6**	9.1*	2.8**
10	6	444	42	13	13.3**	23.6**	3.2*
10	8	400	124	5	32.3**	24.0**	24.8**
0	9	211	87	68	73.4	2.0	1.3
5	9	137	35	4	28.4**	17.9**	8.7**
10	9	238	53	3	19.3**	20.4**	17.7**

* Significant at the 5% level

** Significant at the 1% level

† Number of larvae transferred after treatment

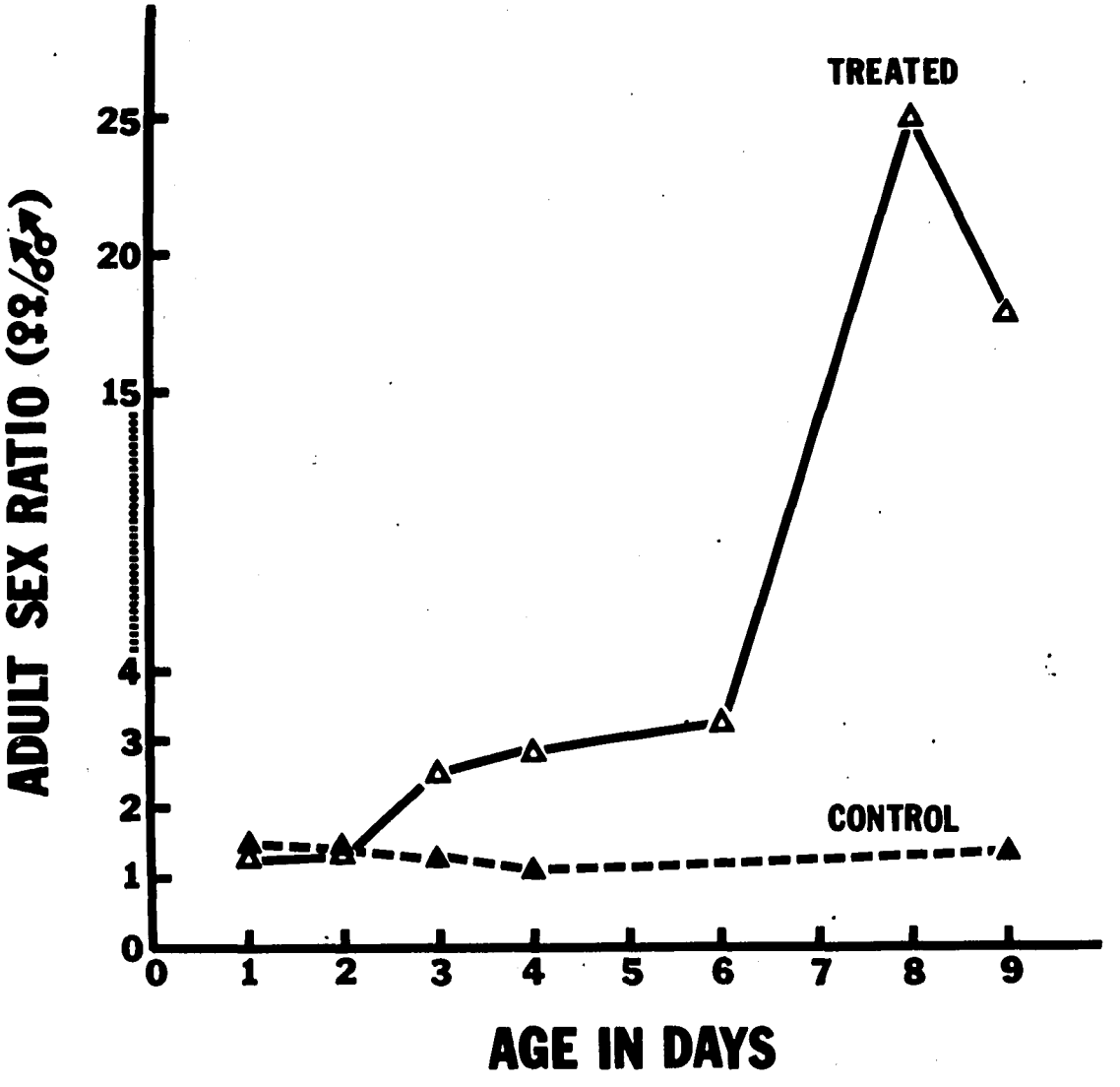


Figure 2. - Effect of actinomycin D on the adult sex ratio (♀♀/♂♂) after treating stock 2 larvae of different ages. (Cross: $\underline{M}/\underline{bb^1}\ \underline{B}\ \underline{oc} \times \underline{bb^1}\ \underline{B}\ \underline{oc}/Y$). 1 and 2-day old larvae treated with 5 µg/ml and all other ages with 10 µg/ml. Data taken from Table 2.

days 4 and 6; rises sharply to 25:1 when 8-day old larvae are treated; and remains at about this level on day 9.

The subsequent experiments were designed to elucidate the genetic basis for the preferential killing by actinomycin D on stock 2 males. Since the results with the wild-type stock indicate that maleness as such does not result in a detectable differential sensitivity to actinomycin D, the possible involvement of the markers carried by stock 2 was investigated. Stock 2 males are hemizygous for the X-limited markers $\underline{sc}^{S1}$ $\underline{sc}^{8}$, B, In49, oc ptg for which the females are heterozygous. Both males and females are heterozygous for the mutant $\underline{bb}^{1}$ (in this stock the normal allele $\underline{bb}^{+}$ is in the X chromosome in females and in the Y in males). Since "late" male larvae, i.e., those close to pupation, were found to be most sensitive to the lethal effect of actinomycin D, all subsequent experiments treated larvae 8 or 9 days of age. In each experiment the test of the effectiveness of a given concentration of actinomycin D is indicated by the percentage of treated larvae that subsequently eclose and by the percentage of abnormal flies.

The first experiment tested whether the hemizygosity for the Bar marker and/or the inversions is responsible for the high sensitivity of stock 2 males to actinomycin D. Stock 3 (M/Basc ♀♀ × Basc/Y ♂♂) has these markers (the InS in Basc is very similar to the In49 in stock 2) and also $\underline{w}^{a}$. Stock 3 tests the effect of actinomycin D on the relative survival of males hemizygous for the Basc chromosome as compared to the females heterozygous for this chromosome and for the Minute-containing X of stock 2 females. As can be seen in Table 3, treatment of 8-day old larvae with 20 µg/ml actinomycin D had no detectable effect on the survival of Basc/Y males as compared to the M/Basc females. (The third type

TABLE 3

Effect of actinomycin D (20 µg/ml) on the viability of Basc/Y males as compared to M/Basc females. Cross: M/Basc × Basc/Y. 8-day old larvae treated

	No. Larvae	Number Eclosing			% Eclosing	% Abnormal Adults
		Basc/Y ♂♂	M/Basc ♀♀	Basc/Basc ♀♀		
Control	601	158	145	169	78.5	5.9
Treated	1165	189	153	215	47.8*	17.6*

* Significant at the 1% level.

TABLE 4

Effect of actinomycin D on the viability of males hemizygous as compared to females heterozygous for ocelliless. Cross: +/oc × oc/Y. 8-day old larvae treated. 0 = Control

Act. D µg/ml	No. Larvae	Number Eclosing				% Eclosing	% Abnormal Adults
		+/oc ♀♀	oc/Y ♂♂	oc/oc ♀♀	+/Y ♂♂		
0	625	149	111	119	130	81.4	3.7
30	1054	171	84*	100	150	47.9**	38.2**
50	1491	99	49	68	95	20.9**	35.7**

* 149/111 and 171/84 differ significantly at the 5% level.

** Significant at the 1% level.

of offspring, Basc/Basc females, was just as abundant as either of the other types). That the concentration of actinomycin D used constitutes an effective dose is shown by the significantly reduced percentage of eclosion of all progeny and by the significantly increased percentage of abnormal flies produced as compared to the control. It is concluded, therefore, that the Bar mutant and the inversions in hemizygous condition are not responsible for the high sensitivity of stock 2 males to actinomycin D.

The possibility that hemizygosity for the ocelliless and pentagon mutants is the cause of the high sensitivity of stock 2 males relative to females heterozygous for these markers was tested by treating 8-day old larvae generated by cross 4 (+/oc ♀♀ × oc/Y ♂♂) with 30 or 50 µg/ml actinomycin D (Table 4). 30 µg/ml treatment resulted in a significant increase in the ratio of +/oc females to oc/Y males. This sex ratio (2.0), however, is clearly smaller than that (17.7–24.8) observed using comparably aged stock 2 individuals. Moreover, no detectable difference was found between the experimentals treated with 50 µg/ml and the control sex ratio (the survival of all four types of progeny is similarly affected). It is concluded, therefore, that ocelliless and pentagon are not responsible for the preferential killing of stock 2 males by actinomycin D.

The preferential killing might be caused by a preferential inhibition of the bb^+ allele, in the Y chromosome of the male as opposed to the bb^+ allele in the X chromosome of the female. This effect would allow the bb^1 in the X of the male to express itself relatively more frequently than the bb^1 mutant in the female. (Such a preferential inhibition of a locus in the Y chromosome could be brought about, for example, by a relatively greater affinity of the Y chromosome than the X for actinomycin D.

Since stock 5 males (bb$^+$ B oc/Y) differ from stock 2 males only in having a bb$^+$ gene instead of bb^1 in the X chromosome, they can be used to test the involvement of bobbed lethal in the preferential killing. To have the same basis for comparing the viability of males to females as in stock 2, stock 5 males have to be compared to females, M/bb$^+$ B oc, which carry the M-containing X of stock 2. When the stock, M/bb$^+$ B oc ♀♀ × bb$^+$ B oc/Y ♂♂, was constructed, only two of three expected types of progeny -- the parental types -- were viable. The absence of the homozygous Bar daughter indicated the presence (not mentioned in the stock list) of a "lethal" in the stock 5 bb$^+$ B oc X chromosome. Moreover, a cross of stock 5 males to attached-X females without a free Y (from stock 9) showed that the males are viable in the XO condition. This finding indicated the presence of a Female lethal (Fl) in the X chromosome of stock 5 males, similar to the Fl mutant discovered by Muller and Zimmering (1960), which has a killing effect on females (ranging from no dominance to complete dominance depending on the presence of modifiers in the rest of the genome) but no detectable effect on males. Stock 5 will now be referred to as M/bb$^+$ B (Fl?) oc ♀♀ × bb$^+$ B (Fl?) oc/Y ♂♂.

The survival of bb$^+$ B (Fl?) oc/Y males relative to M/bb$^+$ B (Fl?) oc females was tested by treating 8 or 9-day old stock 5 larvae with 30 or 10 µg/ml of actinomycin D. The results, summarized in Table 5, clearly show that the male is killed preferentially by actinomycin D. (The increase in the sex ratio resulting from treatment with the two concentrations is not significantly different). Since stock 5 males are sensitive to actinomycin D even though they do not have a bobbed lethal allele, it is concluded that the presence of the bb^1 in stock 2 males is not responsi-

TABLE 5

Effect of actinomycin D on the viability of bb^+ B (F1?) oc/Y males as compared to M/bb^+ B (F1?) oc females. 0 = Control

Act. D µg/ml	Age of Larvae (days)	No. Larvae	Number Eclosing ♀♀†	Number Eclosing ♂♂†	% Eclosing	% Abnormal Adults	Adult Sex Ratio ♀♀/♂♂
0	8	202	82	57	68.8	9.4	1.4
10	8	484	105	15	25.4*	30.0*	7.0*
0	9	207	90	48	66.7	5.8	1.87
30	9	338	56	2	17.2*	32.8*	28.0*

† Does not include crossovers -- 2 B/B ♀♀ and 1 B/Y ♂

* Significant at the 1% level

ble for the preferential killing of these males by actinomycin D. The $\underline{bb^+}$-carrying male, however, seems to be less sensitive than the $\underline{bb^1}$-carrying male, since the sex ratio of stock 5 (7:1) is significantly lower than that of stock 2 (25:1) when 8-day old larvae are treated with 10 µg/ml actinomycin D.

A genetic factor common to stock 2 and stock 5 males may be causing the actinomycin D "sensitivity". Since the preceding experiments ruled out the involvement of B, $\underline{sc^{S1}}$ $\underline{sc^{8}}$, In49, oc ptg -- markers present on both actinomycin D-"sensitive" X's -- the possible involvement of a Female lethal gene, probably present in the chromosome of the stock 5 male, became an important consideration. Does the stock 2 male X chromosome also have an Fl gene, whose presence, however, is masked by the bobbed lethal?

The following crosses were performed to test for the presence of a Female lethal in the stock 2 $\underline{bb^1}$ B oc X chromosome and to confirm the presence of the Fl gene in the stock 5 $\underline{bb^+}$ B (Fl?) oc X:

1) $\underline{M/bb^+}$ B (Fl?) oc ♀♀ × $\underline{bb^1}$ B oc/Y ♂♂. No homozygous Bar daughters, $\underline{bb^+}$ B (Fl?) $\underline{oc/bb^1}$ B oc, were obtained; suggesting either that the $\underline{bb^1}$ B oc X chromosome carries an allele of Fl which acts as a recessive lethal and kills the homozygous Bar daughter, or that the suspected Fl in the $\underline{bb^+}$ X chromosome acts as a recessive lethal in the presence of a stock 5 M-containing X and as a dominant lethal in the presence of a stock 2 $\underline{bb^1}$-containing X.

2) $\underline{M/bb^1}$ B oc and $\underline{M/bb^+}$ B (Fl?) oc ♀♀ × In49 Fl v g/Y ♂♂ -- stock 6 males carrying the Fl of Muller and Zimmering. No heterozygous Bar daughters were obtained from either cross. This result is considered

to confirm the presence of an Fl allele in the bb^+ B (Fl?) oc X chromosome. The result also suggests the presence in the stock 2 male X chromosome of an allele of the known Female lethal, although the possibility still remains that the known Fl acts as a completely dominant lethal in combination with the bb^1 B oc X chromosome.

3) An Fl gene present in the bb^1-containing X chromosome may be detected if the bobbed lethal masking the presence of Fl is removed by crossing over. For this reason, M/bb^1 B oc females were crossed to stock 8 males, y B ct^{ns}/Y, and the F_1 homozygous Bar virgin females, bb^1 B oc/y B ct^{ns}, crossed to bb^1 B oc/Y males. Since Fl^s, an allele of Fl, was mapped at position 19.1 (Zimmering and Muller 1961), the markers ct^{ns}, at 20, and oc, at 23, (as well as the yellow marker) were used to detect the reciprocal crossover products in B ct^{ns}/Y and y B oc/Y males. One male of each type was obtained and stocks were started by crossing each male to Y/y f := (attached-X) females. Sons of these males were then tested for the presence of bb^1 by crossing them to attached-X females without a free Y chromosome (stock 9). No XO sons were obtained from the B ct^{ns}/Y fathers whose genotype was, therefore, bb^1 B ct^{ns}/Y; XO sons were obtained from the other crossover male, indicating the absence of bb^1 in his X, y B oc. This chromosome can now be tested for Fl, and is written as y B (Fl?) oc. These males were crossed to stock 2 females, now also represented as possibly having an Fl gene, M/bb^1 B (Fl?) oc and the F_1 scored. No y B (Fl?) oc/bb^1 B (Fl?) oc daughters were found; the progeny consisted of 63 bb^1 B (Fl?) oc/Y ♂♂ and 2 M/y B (Fl?) oc ♀♀. Moreover, when females of M/y B (Fl?) oc type were backcrossed to y B (Fl?) oc/Y no homozygous y B oc females were obtained. These findings, together with the results of the preceding experiments,

support the conclusion that the stock 2 male X chromosome (and the y B oc crossover X) carry an Fl gene. (The poor viability of the females heterozygous for Fl in the above-mentioned result indicates that our Fl allele, just as the Fl of Muller and Zimmering, can have a partially dominant effect). It is suggested that Fl is the common factor in the stock 2 and stock 5 male X chromosomes which may be responsible for the preferential killing of these males by actinomycin D.

To obtain more evidence for the dependence of the actinomycin D preferential killing effect on the presence of the Fl gene, tests were made of the effect of 10 µg/ml actinomycin D on the viability of the following four types of male as compared to the same type of attached-X female (Table 6): 1) the original actinomycin D-"sensitive" stock 2 male with Fl; 2) the male whose X was used to obtain the crossovers; 3) the crossover male without Fl; 4) the complementary crossover male with Fl. Table 6 shows that treatment of older larvae produced a significantly marked increase in the adult sex ratio over the control only in the case of males 1 and 4 -- which have an Fl gene. The treatment of larvae from males 2 and 3 decreased the percentage of eclosion and increased the percentage of abnormal flies as compared to the control values. Although these changes were not at a level which is statistically significant, it is highly likely that actinomycin D did penetrate larval tissues of males 2 and 3, since some of the treated male flies of these types showed the phenocopy erupt, which occurs only much more rarely in control flies. (Note that rough eye could not be scored in hemizygous Bar eyed males, thus decreasing the total phenocopy frequency). These tests offer more supporting evidence that the preferential killing of stock 2 and stock 5

TABLE 6

Effect of actinomycin D (10 µg/ml) on the viability of males with or without the Fl^M. All males were crossed to Y/y f := females

	Type of Male†		Age of Larvae (days)	No. Larvae	No. Eclosing ♀♀	No. Eclosing ♂♂	% Eclosing	% Abnormal Adults	Adult Sex Ratio (♀♀/♂♂)
1.	$\underline{bb^1}$ $\underline{B}$ $\underline{Fl^M}$ $\underline{oc}$/Y	Control	9	364	158	97	70.1	4.7	1.6
		Treated	9	630	175	28	32.2**	6.4	6.3**
2.	$\underline{y}$ $\underline{B}$ $\underline{ct^{ns}}$/Y	Control	8	29	11	11	75.9	9.1	1.0
		Treated	8	126	35	39	58.7	13.5	0.9
3.	$\underline{bb^1}$ $\underline{B}$ $\underline{ct^{ns}}$/Y	Control	8	68	29	30	86.8	6.8	0.97
		Treated	8	79	35	30	82.3	13.8	1.17
4.	$\underline{y}$ $\underline{B}$ $\underline{Fl^M}$ $\underline{oc}$/Y	Control	8	67	18	22	59.7	0.0	0.82
		Treated	8	170	60	5	38.2**	15.4*	12.0**

† Experiments involving males 2, 3 and 4 were done simultaneously

* Significant at the 5% level

** Significant at the 1% level

males by actinomycin D is dependent upon the presence of a Female lethal gene in these males.

In order to determine whether the presence of the F1 of Muller and Zimmering also causes a preferential killing of the males, stock 6 larvae (In49 F1 v g/Y ♂♂ × Y/y w f :=♀♀) were treated with 10 µg/ml actinomycin D. Thus the survival of males carrying the F1 of Muller and Zimmering was being compared to attached-X females very similar (having an additional white marker) to those used in the tests just described (Table 6). The results summarized in Table 7 show that the adult sex ratio in the treated series is not detectably different from that of the control, although the antibiotic produced a significant number of flies with phenocopies. It is concluded that the F1 allele of Muller and Zimmering does not make males sensitive to actinomycin D. Since the newly discovered F1 allele behaves differently (there being no known difference between the alternatives in stock 2 and stock 5), it is designated $F1^{M}$, Female $lethal^{Margulies}$.

F1 kills homozygotes in the egg stage (Muller and Zimmering 1960). Since only 2 (probably nondisjunctional XO males) instead of about 90 yellow larvae hatched from 370 eggs produced by the cross, M/y B $F1^{M}$ oc ♀♀ × y B $F1^{M}$ oc/y^{+} Y ♂♂. $F1^{M}$ probably kills all female homozygotes in the egg stage.

Is the Y chromosome instrumental, either in a beneficial or detrimental way, in the survival of stock 2 and stock 5 $F1^{M}$-containing males under conditions of actinomycin D treatment? The possible involvement of the Y chromosome is suggested by evidence that certain X-limited lethals (Lindsley, Edington, and Von Halle 1960) and the sc^{8-} lethal factor

TABLE 7

Effect of actinomycin D (10 µg/ml) on the viability of In49 Fl v g/Y males as compared to Y/y w f := females. 8-day old larvae treated.

	No. Larvae	No. Eclosing ♀♀	No. Eclosing ♂♂	% Eclosing	% Abnormal Adults	Adult Sex Ratio (♀♀/♂♂)
Control	182	73	75	81.3	4.1	0.97
Treated	339	97	152	73.4	37.3*	0.64

* Significant at the 1% level.

(Hess 1962) are suppressed by the Y chromosome. If the Y helps the survival of $\underline{Fl}^{M}$-containing males treated as older larvae, then the XO male should be even less viable, and the resulting adult sex ratio even higher. If, alternatively, the Y chromosome does not have a saving effect in older larvae, but has such a beneficial effect in younger larvae, this might explain the relatively much smaller killing effect of actinomycin D on young males of stock 2 (3-6 days in age, Table 2). If the latter alternative were true, young XO males would be more sensitive to actinomycin D than young XY males.

$\underline{y}\ \underline{v}\ \underline{bb}$.= females with a Y were obtained as F_1 virgins from the cross of stock 9 attached-X females without a Y to stock 5 males. $\underline{y}\ \underline{v}\ \underline{bb}$.= females with or without a Y chromosome were crossed to $\underline{bb}^{+}\ \underline{B}\ \underline{Fl}^{M}\ \underline{oc}/Y$ (stock 5) males to generate in F_1 stock 5 XY and XO males, respectively. The viability of XY and XO males after actinomycin D treatment could thus be compared to the same type of female. The results of treating 4-day and 8-day old larvae with 10 µg/ml actinomycin D are shown in Table 8. There was no detectable difference in the survival of the XY and XO males when 4-day old larvae were treated and no increase in the adult sex ratio was observed as compared to the control. (It should be noted, by way of comparison, that treatment of 4-day old larvae of stock 2 males gave a 2:1 adult sex ratio when measured against $Y/\underline{y}\ \underline{f}$:= females, and a 2.8:1 sex ratio when measured against stock 2 Minute females. (Table 2). It seems, therefore, that the $\underline{bb}^{1}$-carrying stock 2 male is more sensitive to actinomycin D than the $\underline{bb}^{+}$-carrying stock 5 male when 4-day old larvae are treated. Possibly, the higher sensitivity of the $\underline{bb}^{1}$-carrying male, also observed in the case of 8-day old larvae, may be attributed

TABLE 8

Effect of actinomycin D (10 µg/ml) on the viability of the X0 and XY male carrying bb^{+} B Fl^{M} oc relative to the attached-X female, Y/y v bb .=

	Age of Larvae (days)	No. Larvae	No. Eclosing		% Eclosing	% Abnormal Adults	Adult Sex Ratio (♀♀/♂♂)
			Y/.= ♀♀	XY ♂♂			
Control	4	107	35	43	72.9	3.8	0.81
Treated	4	387	48	53	26.1*	7.9	0.91
Control	8	260	87	93	69.2	4.4	0.94
Treated	8	370	112	25	37.0*	34.3*	4.48*

			Y/.= ♀♀	XY ♂♂			
Control	4	107	35	43	72.9	3.8	0.81
Treated	4	387	48	53	26.1*	7.9	0.91
Control	8	260	87	93	69.2	4.4	0.94
Treated	8	370	112	25	37.0*	34.3*	4.48*

			Y/.= ♀♀	XO ♂♂			
Control	4	128	42	41	64.8	15.7	1.02
Treated	4	368	55	55	29.9*	12.7	1.0
Control	8	142	40	49	62.7	3.4	0.82
Treated	8	465	132	20	32.6*	36.8*	6.67*

* Significant at the 1% level

to a general weakening due to the presence of the additional mutant gene).

A similar, preferential killing of stock 5 XO and XY males occurred when older, 8-day old larvae were treated. It is concluded, therefore, that the actinomycin D-caused preferential killing of older, $\underline{Fl}^{M}$-containing males is independent of the presence of the Y chromosome.

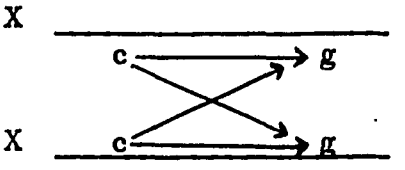
GENERAL DISCUSSION

What mechanism can be suggested to explain the hypothesis that the $\underline{Fl}^{M}$ gene is responsible for the much higher actinomycin D "sensitivity" of males that are hemizygous relative to females that are heterozygous for this gene? (Note that the effect of $\underline{Fl}^{M}$ was never detected in the absence of the markers $\underline{sc}^{S1}$ $\underline{sc}^{8}$, In49, B and oc ptg). Assuming that the actinomycin D-caused preferential killing of males is due to the inhibition of DNA-dependent RNA synthesis, it is highly unlikely that it can be attributed to the inhibition of gene action of the $\underline{Fl}^{M}$ locus itself, for the following reasons: $\underline{Fl}^{M}$ is a harmful mutant (when hetero- or homozygous) in females and probably produces its damage either because it fails to make mRNA or produces a modified mRNA. Assuming that $\underline{Fl}^{M}$ acts in males as in females, the antibiotic should have no effect or a beneficial effect, respectively, in $\underline{Fl}^{M}$ males. Moreover, it is improbable that actinomycin D affects the single locus, $\underline{Fl}^{M}$, frequently enough to account for the observed magnitude of male lethality. It is more likely, therefore, that the inhibition of more than one gene by actinomycin D is involved in the preferential killing of males. What genes can be implicated? It is hypothesized that these are other X-limited genes whose action is already partially inhibited by $\underline{Fl}^{M}$ and

which, when further inhibited by actinomycin ^{D}, cause the death of males. The preferential lethality of $\underline{Fl}^{M}$-containing males relative to heterozygous females may be due to the fact that genes inhibited by $\underline{Fl}^{M}$ are present in pairs in females, only one member of which is expected to be further inhibited by actinomycin D if the antibiotic were present in the nucleus in limited amounts. Our results on phenocopy induction by actinomycin ^{D} (Margulies, submitted for publication) support the assumption that both homologous loci are affected simultaneously by actinomycin D relatively rarely. This conclusion is suggested by the relatively low frequencies of most types of phenocopies obtained, since the phenotypic changes probably result from the inhibition by actinomycin D of both alleles of the gene or genes controlling the development of the particular adult trait. Rough eye, the one phenocopy which occurs in high frequencies (in about 50% of the adults when "late" wild-type larvae are treated with actinomycin D), is a trait which is affected by many autosomal and X-limited loci. Thus the inhibition of any one of the pairs of genes controlling this trait (or combinations of single members of different gene pairs) may result in the rough eye phenocopy.

$\underline{Fl}^{M}$ and the other X-limited genes whose action is hypothesized to be inhibited by $\underline{Fl}^{M}$ can be considered in the light of dosage compensation. One dose of the $\underline{Fl}^{M}$ gene in males should have the same effect as two doses in females if this mutant showed dosage compensation. When dosage compensation does occur modifiers in the X chromosome, referred to as "compensators", are hypothesized (Muller 1950) to inhibit the effect of

other X-limited genes (to be dosage compensated) so that their product is equalized in both sexes. This equalization would be accomplished if compensators, c, in single dose in the X of a male reduced the potential product (1) of a gene being compensated, g, by $\frac{1}{2}$, each of the two doses of c in the two X's of a female reducing the effect of each of the two doses of g by $\frac{1}{2}$, as indicated by the arrows (representing compensative action) in the following diagram.



The effect of actinomycin D on the adult sex ratio of the wild-type stock and the postulated mechanism for the action of $\underline{Fl}^{M}$ alone and in combination with actinomycin D is summarized in Table 9. When wild-type female larvae are treated with actinomycin D, it is expected that most of the time only one of the two alleles of the compensated locus, g, will have the synthesis of its product inhibited by actinomycin D (by a fraction 1/a). In such a case the gene product per individual would be greater in females than in males ($\frac{(1 + a)}{4a}$ is greater than 1/2a, respectively, for all values of "a" greater than 1), assuming that the probability of inhibiting one X-limited g locus in males is the same as that of inhibiting either one of the two g's in females. It might be expected, therefore, that actinomycin D-treated females would survive better than males. Combining the data of 18 experiments with the wild-type stock, the adult sex ratio ($\varphi\varphi/\delta\delta$) in the experimental flies was increased as compared to the control (1.17 and 1.04 respectively), but this was not a statistically significant difference. It is

TABLE 9

A model showing Muller's dosage compensation hypothesis and our postulated effect on X-limited genes of actinomycin D alone, Fl^M alone and Fl^M in combination with actinomycin D. g = dosage compensated X-limited gene. c = dosage compensators in the X chromosome. Act. D = actinomycin D

			Potential Product of g per X	Reduction Factor in Product per X: Due to c's	Due to Fl^M's	Due to Act. D	X	Actual g Product per: Individual	Sex Ratio (♀♀:♂♂): Approximate Expected	Typical Found
Wild-type	♀	+ c g	1	(½) (½)	-- --	--	½	½	1:1	1.04:1
		+ c g	1	(½) (½)	-- --	--	½			
	♂	+ c g	1	(½) --	-- --	--	½	½		
	♀	+ c g + Act. D	1	(½) (½)	-- --	(1/a)	1/4a	(1 + a)/4a	>1:1	1.17:1
		+ c g + Act. D	1	(½) (½)	-- --	--	1/4			
	♂	+ c g + Act. D	1	(½) --	-- --	(1/a)	1/2a	1/2a		
Fl^M	♀	Fl^M c g	1	(½) (½)	(1/f) (1/f)	--	$1/4f^2$	$1/2f^2$	0:1	0:1
		Fl^M c g	1	(½) (½)	(1/f) (1/f)	--	$1/4f^2$			
	♀	Fl^M c g	1	(½) (½)	(1/f) --	--	1/4f	1/2f	1:1	1.3:1*
		+ c g	1	(½) (½)	(1/f) --	--	1/4f			
	♂	Fl^M c g	1	(½) --	(1/f) --	--	1/2f	1/2f		
	♀	Fl^M c g + Act. D	1	(½) (½)	(1/f) --	(1/a)	1/4fa	(1 + a)/4fa	>>1:1‡	25:1†
		+ c g + Act. D	1	(½) (½)	(1/f) --	--	1/4f			
	♂	Fl^M c g + Act. D	1	(½) --	(1/f) --	(1/a)	1/2fa	1/2fa		

* Based on all controls in Table 2.

† From Table 2.

‡ Much greater than 1:1

possible, however, that because of the equal killing of males and females due to the inhibition of genes in the autosomes -- which contain about 75% of the total nuclear chromosomal material (see Herskowitz and Muller 1954) -- it may be difficult to detect any preferential killing of wild-type males. Moreover, it is not known how much of the killing by actinomycin D is due to its inhibition of energy metabolism (Lazzlo et al. 1966) which probably affects females and males equally and which, being unrelated to the inhibition of RNA synthesis, may further obscure a possible differential effect on X-limited genes in males and females.

As shown in Table 9, it is suggested that in the case of $\underline{Fl}^{M}$-containing individuals g genes are inhibited by $\underline{Fl}^{M}$, the product of each g being reduced by a fraction (1/f). This results in the death of females with two doses of the mutant, but permits viability of males and females with one dose. (The actual g product per individual for a compensated locus inhibited by 2 doses of $\underline{Fl}^{M}$, $1/2f^2$, is smaller than 1/2f, calculated for males and females with one $\underline{Fl}^{M}$, for all values of "f" greater than 1).

It is suggested that treatment of females heterozygous for $\underline{Fl}^{M}$ with actinomycin D permits much greater survival than treatment of males hemizygous for $\underline{Fl}^{M}$, because in females only one allele of a dosage compensated and $\underline{Fl}^{M}$-inhibited pair of genes is further inhibited by actinomycin D (by fraction 1/a) -- assuming again that the concentration of actinomycin D is limiting. The gene product per individual female $\left(\frac{1 + a}{4fa}\right)$ is greater than that per individual male (1/2fa) for all values of "f" and "a" greater than 1. Therefore, the inhibition by

actinomycin D of the one g in the one X of the male results in a gene product which is too small for survival, thus killing the male. Since $\underline{F1}^{M}$ is hypothesized to affect the activity of a number of X-limited genes, an $\underline{F1}^{M}$-carrying male might be killed any time one of those unpaired genes is inhibited by actinomycin D. It is easy to see, therefore, how actinomycin D could cause a preferential killing $\underline{F1}^{M}$-carrying males relative to heterozygous females.

Males carrying the F1 allele of Muller and Zimmering are not sensitive to actinomycin D. It may be speculated that F1 inhibits the action of fewer genes than $\underline{F1}^{M}$, the chances of an inhibited locus being affected by actinomycin D being so small that the F1-carrying males are not detectably more sensitive to actinomycin D than treated $\underline{F1}^{+}$ stocks. The discovery of $\underline{F1}^{s}$ (Zimmering and Muller, 1961) -- which causes females to be sterile when homozygous and to die when heterozygous with F1 -- supports the existence of multiple alleles at the F1 locus. It is suggested that the wild-type allele of the Female lethal mutants, $\underline{F1}^{+}$, may be a gene which regulates the activity of other X-limited genes, and that F1 mutants (recessive to the wild-type allele in the absence of modifiers) have resulted in an excessive inhibition of these genes.

The preferential killing by the combination of $\underline{F1}^{M}$ and actinomycin D affects mainly older male larvae. Such an effect might be explained by differential gene transcription (see Margulies, submitted for publication), that is, if many of the genes hypothesized to be inhibited by $\underline{F1}^{M}$ were activated later in larval development.

SUMMARY

Drosophila melanogaster males of two exceptional stocks were shown to be preferentially killed by actinomycin D; the adult sex ratio (♀♀/♂♂) rising as high as 25:1 when older larvae were treated. This actinomycin D-induced effect is independent of the presence of various genetic markers and of the Y chromosome, but is dependent on the presence of a non-dosage-compensated gene, *Female lethal*^{Margulies} (*Fl*^{M}), detected in the X's of both exceptional stocks. This gene behaves allelic to Muller and Zimmering's *Fl* although males carrying the latter allele are not preferentially sensitive to actinomycin D. The preferential killing of hemizygous *Fl*^{M} males (relative to heterozygous *Fl*^{M} females) is hypothesized to result from the inhibition by *Fl*^{M} of other X-limited, dosage compensated, genes which cause lethality when further inhibited by actinomycin D.

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AUTOBIOGRAPHICAL STATEMENT

I was born in Poland in 1930, and emmigrated to the U. S. A. in 1947 where I became a citizen in 1954. I graduated from Yonkers High School in 1949 and began to attend New York University with a full-tuition scholarship from The Educational Foundation for Jewish Girls. I was married in 1951 to Joseph Margulies, now a CPA; we have an 11-year old son. In 1954 I enrolled in the Evening Session at Hunter College where I majored in Physiology and minored in Chemistry, and received the A.B. degree cum laude in 1961.

In 1962 I entered the M.A. program in Biology at Hunter College, transferring in 1963 to the Ph.D program in Biology at The City University of New York. I majored in Genetics and did the thesis research in this area under the direction of Dr. I. H. Herskowitz.

While preparing for the Ph.D., I received financial assistance in the form of a one-year Fellowship from the Sloan Foundation, a two-year Fellowship from The City University of New York, and by working as a part-time research assistant for Dr. Herskowitz.

I taught "Principles of Genetics" in the School of General Studies at Hunter College in Spring 1967, and am currently teaching "Principles of Biology", recitation and laboratory, in the Day Session of Hunter College.

I am a member of the Genetics Society of America and Phi Sigma.