STUDIES ON THE SEX-SPECIFIC LETHALS OF *DROSOPHILA MELANOGASTER*. II. FURTHER STUDIES ON A MALE-SPECIFIC LETHAL GENE, MALELESS[1 2]

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ABSTRACT

Effects of a second chromosome male-specific lethal gene, maleless (*mle*), of *Drosophila melanogaster* were further studied. It was shown that, although no maternal effect was seen with respect to the male-specific lethality, the lethal stage was influenced by whether parental females were homozygous or heterozygous for *mle*. Thus, in the former *mle/mle* males died mostly in the late third instar larval stage, while in the latter practically all males survived to the pupal stage. In the dying *mle/mle* male pupae complete differentiation of adult external head and thorax structures was often observed but that of abdominal structures was incomplete forming only a few segments in most cases. Imaginal discs from third instar *mle/mle* male larvae which were produced by *mle/mle* mothers and were destined to die as larvae were able to differentiate into adult structures upon transplantation into normal third instar larval hosts.——A somewhat elaborated version of the previously presented hypothesis (FUKUNAGA, TANAKA and OISHI 1975) was discussed as to the possible presence of a class of sex-specific lethals which are not related to the process of primary sex differentiation.

ABNORMAL sex-ratio conditions due to zygotic selection may provide a unique means to attack the general problem of sex differentiation. Several cases of such abnormal sex-ratios have been reported in *Drosophila melanogaster*. For example, COLAIANNE and BELL (1972) reported that a sex-linked maternally influenced male-lethal gene, sonless (*snl*), interacts with sex-transforming genes, transformer (*tra*) and doublesex (*dsx*). Thus, progeny flies of *snl/snl* mothers are affected to various degrees depending on their phenotypic sex. OISHI (1971) proposed that a maternally inherited abnormal sex-ratio producing agent produces an exotoxin-like substance, *androcidin*, which interacts with developmental systems that produce biochemical differences between male and female sexes, although they may not be directly related to the primary sex differentiation (MIYAMOTO and OISHI 1975).

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In our previous paper (FUKUNAGA, TANAKA and OISHI 1975), we have reported on a male-specific lethal gene, maleless (*mle*), of *D. melanogaster* and have characterized it with the above context in mind. The gene is a second chromosome recessive located at about 55, and has no maternal effect with respect to the male-specific lethality. It interacts neither with *tra* nor with *dsx*, thus only single *X* individuals are killed regardless of their phenotypic sex. The gene is unique among abnormal sex-ratios in its late lethal effect, and thus we have examined it further focusing on this late lethal condition.

MATERIALS AND METHODS

Mutant stock: Origin, maintenance, and the elementary genetics of the *mle* stock were described previously (FUKUNAGA, TANAKA and OISHI 1975). During the present study the stock was maintained by crossing *mle/mle* females with *mle/SM1(Cy)* males. As previously experienced, the fecundity of *mle/mle* females gradually decreased when the stock was maintained in this manner. Whenever necessary, therefore, *mle/SM1(Cy)* females and males were crossed and the stock re-established (Figure 1). Care was taken to select flies as often as possible which showed good segregation ratios, so as not to accumulate deleterious genes in the *mle*-carrying chromosome.

Flies were reared at 24 ± 1° on a sucrose-yeast-agar medium (per liter of water: sucrose 55 g, dry powdered yeast 35 g, agar 19 g, with 4 ml of propionic acid added) or on a similar medium to which was added commercial grape juice to color the medium when eggs were counted.

Viability studies—egg collection: For the viability studies two kinds of crosses, cross 1 and cross 2, were made as shown in Figure 1. In each cross a young virgin female (2–4 days old) was individually crossed with a single male, the pair transferred to new culture bottles everyday, and the number of eggs deposited was counted. The number of eggs which hatched was determined 24 hr after the parental flies were removed. The number of pupae and adults were recorded as they appear until the days 12–13 and 18, respectively. Results obtained for each brood were summed and the total numbers were shown in the Table. First broods were omitted from the data since they often included many apparently unfertilized eggs.

Viability studies—larva collection: Similar crosses were made as above but this time 5-pair matings were made instead of the single-pair, and parental flies were transferred to new culture bottles twice a day, once in the morning and the other in the evening, for several days. Those animals still in the third instar larval stage were collected between the days 7 and 8, sexed by visually determining the size of the gonads, placed in fresh culture bottles, and the numbers of pupae and adults were counted daily until the day 19–21.

Histological examination of dead pupae: Dead pupae were placed on microscopic slides, carefully removed from the pupal case, mounted in Faure's water soluble mounting medium

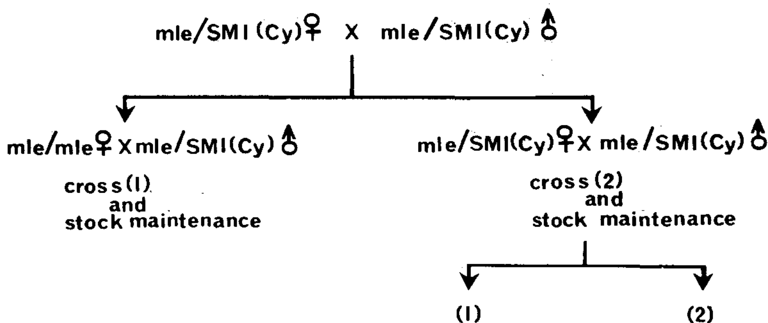

FIGURE 1.—Crosses for stock maintenance and experiments.

(per 50 ml of distilled water: glycerol 20 g, gum arabic powder 30 g, chloral hydrate 50 g), and examined under a compound microscope.

Imaginal disc transplantation: Crosses were made as described above. Larvae were collected between days 7 and 9 and sexed. Imaginal discs were obtained by the methods described by URSPRUNG (1967), and transplanted into third instar (72–96 hr) wild-type larvae. Microinjection pipettes were prepared as described by GURDON (1974), and the injection apparatus was a modification of RIZKI (1953).

At the same time salivary glands were processed for the chromosome examination so as to make certain that the larvae are *mle/mle* and not *mle/SM1(Cy)*.

RESULTS

1. *Maternal effects on the lethal stage of* mle/mle *male progeny.*

Table 1 shows the viability at various developmental stages of progeny from *mle/mle* mothers (cross 1, sum of 13 single-pair cultures totaling 66 single-day broods) and from *mle/SM1(Cy)* mothers (cross 2, sum of 8 single-pair cultures totaling 43 broods). Since the *mle* stock had been maintained as described in METHODS and since these particular crosses were made as shown in Figure 1, the general genetic background of flies in two crosses may be expected to be essentially the same. If there had been no deleterious genes other than *mle* and *Cy* (homozygous lethal) involved, cross 1 should have produced 2335 adults (75% of 3113), and cross 2 1003 (62.5% of 1605). It thus appears as if many heterozygous and homozygous *mle* males and females died for reasons other than being *mle/mle* or *Cy* homozygotes.

In cross 1 the segregation in adults is quite good ($0.5 > P > 0.3$), thus we may expect that those unknown reasons affected all the phenotypes equally. We can, then, expect some 555 *mle/mle* males died during the development, and simple calculation shows that at least more than one-half of them died before pupation.

TABLE 1

Maternal effects of the mle *gene on the lethal stage of* mle/mle *male progeny*

No. eggs	No.* hatched	No.† pupated	No.‡ eclosed	Segregation in adults (Expected ratio) *Cy* ♀	*Cy* ♂	+ ♀	+ ♂
Cross 1: *mle/mle* females × *mle/SM1(Cy)* males							
3113	2583	1894	1711§	573	527	565	0
(1.00)	(0.83)	(0.61)	(0.55)	(1	1	1	0)
Cross 2: *mle/SM1(Cy)* females × *mle/SM1(Cy)* males							
1605	1323	1074	811‖	301	357	133	0
(1.00)	(0.82)	(0.67)	(0.51)	(2	2	1	0)

* Determined 24 hr after parental flies were removed.
† Total number of those pupated by the day 12–13 after egg collection.
‡ Total number of adults emerged by the day 18 after egg collection.
§ Out of 1711, 46 flies (39 females and 7 males) died immediately after emergence and thus their phenotypes could not be determined.
‖ Out of 811, 20 (17 females and 3 males) died immediately and their phenotypes could not be determined.

In cross 2, although the segregation in adults here is not good ($P < 0.01$), the fact that 67%, which is much more than 62.5% as calculated above, pupated strongly suggests that most *mle/mle* males pupated since *Cy* homozygotes rarely pupate, if ever.

It was noted that there were many mature-looking larvae crawling around even on the day 10 in the progeny from *mle/mle* mothers, while there were few in those from *mle/SM1(Cy)* mothers on that late date. The same crosses as in Table 1 were made and successive half-day broods were taken. Those still in the larval stage on the days 7–8 were collected and their development followed (Table 2).

A total of 78 such larvae were recovered from *mle/mle* mothers (cross 1, Exp. I-1), of which 65 were males. Only 18 male larvae pupated and none eclosed. It was observed that for the first 3 days after collection none of the larvae pupated and only one did so on the 4th day after collection. All the larvae were alive for at least 5 days after collection of the larvae. Segregation in adults obtained from the cross was good (footnote ||), thus, although egg counts were not made, the following estimates would not be too unreasonable. Some 86 *mle/mle* males died due to the lethal action of *mle* itself, of which some 21 (24%) died before the days 7–8, 65 (76%) were still alive as mature third instar larvae, 18 (21%) pupated, and none eclosed. In a repeat experiment (cross 1, Exp. II-1) 61 larvae were recovered of which 56 were males. Thirty-seven of them pupated and 2 eclosed (both were *mle/SM1(Cy)* males). Although segregation in adults (footnote ¶) was not as good as that of Exp. I-1 (footnote ||), the deviation is within the limit of statistical expectation ($0.1 > P > 0.05$). We may then expect, as above, some 180 *mle/mle* males, of which only 56 (31%) were collected as mature-looking larvae and 37 (21%) pupated. The discrepancy between the number of surviving larvae at the time of collection in the two experiments, I-1 and II-1, may be the reflection of unknown environmental effects.

In cross 2, Exp. I-2, total of 48 larvae were recovered from *mle/SM1(Cy)* mothers of which 42 were males. Since the segregation in adults was good (footnote **) we may expect some 50 *mle/mle* males, of which 42 pupated and none eclosed. In this case 37 out of 42 pupated did so within 3 days after larva collection and 41 within 4 days. Thus 8 *mle/mle* males, the difference between the recovered 42 and the expected 50, would probably have already pupated before the time of larva collection. Essentially the same results were obtained in a repeat experiment, Exp. II-2. Here, 109 larvae were recovered and of which 107 were males. Twenty-three larvae were sacrificed for salivary gland chromosome examination and all proved to be *mle/mle* males. Eighty-four male larvae were followed for their development. Numbers in parentheses for Exp. II-2 were thus so corrected assuming that the 23 sacrificed male larvae would show the same development at the same rate as those 84 male larvae.

Salivary gland chromosomes of such larvae were further examined in separate experiments. In cross 2, 39 larvae were examined of which 36 were *mle/mle*, 2 were uncertain, and 1 was *mle/SM1(Cy)*. Many male larvae (11/17) from *mle/mle* mothers (cross 1) produced preparations of salivary gland chromo-

TABLE 2

Viability of mle/mle *male larvae in subsequent developmental stages and differentiation of various structures in two kinds of crosses*

Experiments I–1 and I–2 were carried out simultaneously with parental flies obtained as shown in Figure 1, so were II–1 and II–2.

Exp.	No. male larvae/ Total No. collected*	No.† pupated	No.† eclosed	No. showing differentiation of various structures: Prepupal cuticle formation	Head eversion	Wing eversion	Leg eversion	Eye pigmentation	Thorax formation	Abdomen‡ formation	Genitalia§ formation
				Cross 1: *mle/mle* females × *mle/SM1(Cy)* males							
I–1‖	65/78 (0.76)‡‡	18 (0.21)	0	17 (0.20)	4 (0.05)	—¶¶	—	0	1 (0.01)	—	—
II–1¶	56/61 (0.31)	37 (0.20)	2§§	35 (0.19)	27 (0.15)	32 (0.18)	32 (0.18)	5 (0.03)	5 (0.03)	5 (0.03)	1 (0.01)
				Cross 2: *mle/SM1(Cy)* females × *mle/SM1(Cy)* males							
I–2**	42/48 (0.84)	42 (0.84)	0	42 (0.84)	27 (0.54)	—	—	26 (0.52)	26 (0.52)	—	—
II–2††	84/86‖‖ (1.11)	80 (1.05)	0	80 (1.05)	46 (0.60)	77 (1.01)	77 (1.01)	36 (0.47)	35 (0.46)	25 (0.33)	12 (0.16)

* Larvae collected on the day 7–8 after egg collection.

† Total number counted by the day 19–21 after egg collection.

‡ The abdomen formation was incomplete in all cases. The number includes all those with only partial abdomen formation. See text for further explanation.

§ Always found embedded in the abdomen.

‖ From the cross, total of 261 flies eclosed. Of these 2 females and 2 males died immediately and thus their phenotypes could not be determined. Segregation in 257 adults was: 89 *Cy* females, 77 *Cy* males, 91 + females, 0 + males. $0.7>P>0.5$.

¶ 542 adults emerged, of which 2 females and 1 unknown sex died immediately. 30 pupae did not eclose. Segregation in 539 adults in the above order was: 171, 164, 204, 0. $0.1>P>0.05$.

** 269 adults emerged, of which 7 females and 12 males died immediately. Segregation in 250 adults was: 106, 100, 44, 0. $0.7>P>0.5$.

†† 487 adults emerged, of which 3 females died immediately. 18 pupae did not eclose. Segregation in 484 adults was: 189, 216, 79, 0. $0.1>P>0.05$.

‡‡ For numbers in parentheses, see text for explanation.

§§ Both were *mle/SM1(Cy)* males.

‖‖ 109 larvae were collected, of which 107 were males. 23 larvae were sacrificed for salivary gland chromosome examination and all proved to be *mle/mle* males. 84 male larvae were followed for their development. See text for further explanation.

¶¶ Not examined.

somes which were too poor to yield any results as previously experienced (FUKUNAGA, TANAKA and OISHI 1975), but when preparations were good they were always *mle/mle* (6/17).

2. *Differentiation of various structures in* mle/mle *males.*

On the right half of Table 2 is shown the extent of differentiation of various structures observed in the *mle/mle* male pupae which never eclosed. These dead *mle/mle* pupae showed in most cases a development more advanced than a mere prepupa. In cross 1, Exp. II-1, about 30 individuals out of 35 pupated showed the head, wing, and leg eversion. Among these, 5 pupae proceeded further in development and produced nearly complete adult head and thoracic external structures, but even in these the development of adult abdominal structure was incomplete, forming only a few abdominal tergites. It was also noted that the frequency of head eversion was lower than that of wing and leg eversion. This becomes more evident in Exp. II-2.

Out of 80 *mle/mle* males which pupated in cross 2, Exp. II-2, 77 showed the wing and leg eversion or more advanced development, while only 46 showed the head eversion. A typical example showing nearly complete adult thorax formation and incomplete abdomen formation but no head eversion with larval mouth armature still attached is shown in Figure 2a. In most extreme cases, though rare, well-pigmented eyes were seen through the thorax but the pupae had the larval mouth armature attached and no head eversion observed. Adult abdominal tergites were again very poorly formed (Figures 2c and 2d). In essentially all

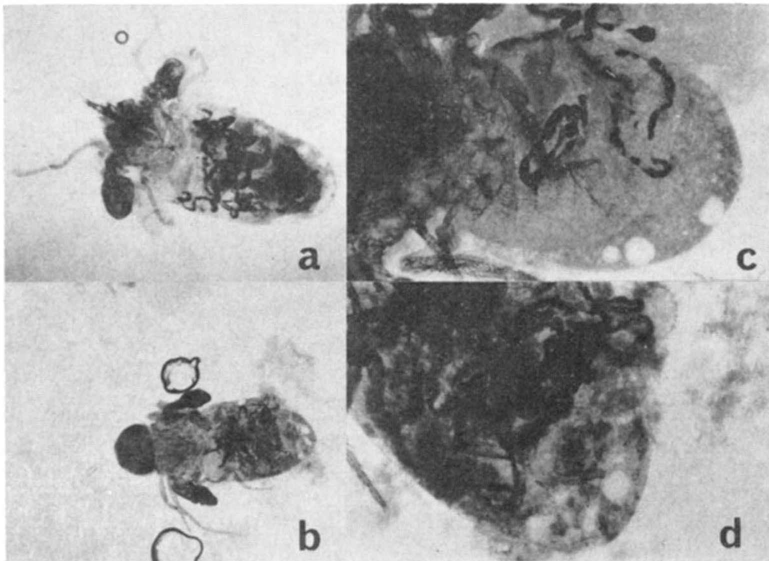

FIGURE 2.—Examples of dead *mle/mle* male pupae showing the best differentiation of various structures. (a) A pupa with complete thorax, incomplete abdomen, and no head. Note that larval mouth armature is still attached. (b) A pupa showing nearly complete adult structure differentiation except for the abdomen. Note the presence of external genital structure embedded in the abdomen. (c) An abdomen showing a partial formation of tergites. (d) An abdomen showing the embedded external genital structure.

cases only the first few tergites were formed at the best. Twelve pupae had external adult genitalia embedded in the abdomen (Figure 2d). This structure was never found on the surface of abdomen.

Homozygous *mle* male larvae from *mle/mle* mothers were smaller in size and remained so even on the day as late as 10 or more, and their salivary gland chromosomes became degraded as the larvae became older. Imaginal discs were also small in these larvae.

3. *Developmental capability of imaginal discs from* mle/mle *male larvae produced by* mle/mle *mothers.*

A possibility that the maternal effect involved in the actual lethal stage as demonstrated above may also be acting on the developmental capability of imaginal discs from the *mle/mle* male larvae produced by *mle/mle* mothers was examined by transplantation of discs into wild-type third instar larval hosts. This was necessary since there was a possibility that those pupae (Table 2, Exp. I-1, II-1) were *mle/SM1(Cy)* males in view of the fact that there were 2 adults (*mle/SM1(Cy)* males) emerged in the cross, although the similarity in the extent of adult structure differentiation in crosses 1 and 2 and the results of salivary gland chromosome examination seem to prove that those males were *mle/mle*. For another reason of this transplantation experiment, see DISCUSSION.

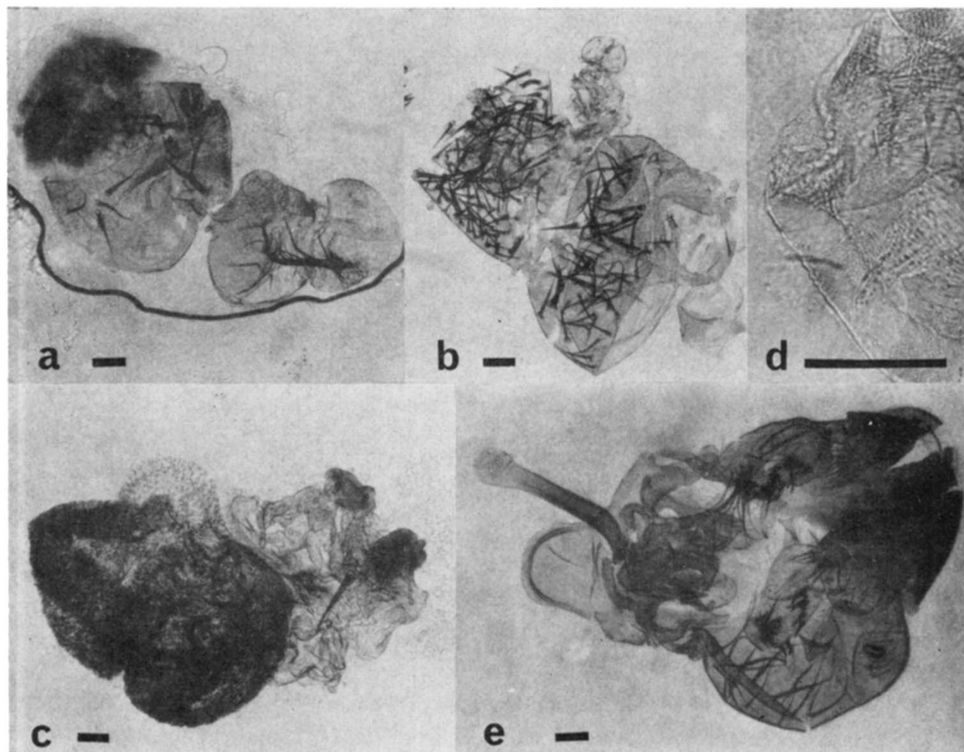

FIGURE 3.—Differentiation of *mle/mle* male imaginal discs upon transplantation into wild-type larval hosts. (a) eye-antenna. (b) leg. (c) wing. (d) haltere. (e) genitalia. Scale indicates 50 μ.

Figure 3 shows that all the major imaginal discs examined (eye-antennal, leg, wing, haltere, and genital) differentiated completely to typical adult structures. That those larvae from which imaginal discs were taken were *mle/mle* males was proved by salivary gland chromosome examination done on the same individuals.

Wild-type third instar female larvae as well as male larvae were used as hosts. The differences in sex of the host appeared not to affect the extent of the disc differentiation.

DISCUSSION

The male-specific lethal gene, *mle*, acts late in the development and maternal effect is involved with respect to the actual lethal stage. Thus, when the female parents are *mle* heterozygotes, practically all the *mle/mle* males survived to the pupal stage and about one-half of these showed nearly complete differentiation of adult surface structures except for the abdomen. Further, although pupation was infrequent and the differentiation of adult structures was rarely seen in the *mle/mle* progeny of the homozygous *mle* mothers, all the larger imaginal discs examined showed complete differentiation upon transplantation into wild-type host larvae. These results indicate that the imaginal discs are potentially normal and suggest that the cells or tissues responding to the action of *mle* are either larval or imaginal non-epidermal.

The head and thorax structures of *mle/mle* males were always nearly complete when formed. In contrast, the abdominal structure was not only less frequently formed but was incomplete. The number of abdominal histoblast cells remains the same throughout the larval period but starts to increase rapidly immediately after the pupariation (GARCIA-BELLIDO and MERRIAM 1971; GUERRA, POSTLETHWAIT and SCHNEIDERMAN 1973). Whether it is this rapid cell proliferation in abdominal histoblasts that is blocked in the *mle/mle* males or whether it is the process of differentiation into imaginal tergites and sternites that is blocked is yet to be studied. The frequent failure of head eversion in the *mle/mle* males also requires further investigation.

In the previous paper (FUKUNAGA, TANAKA and OISHI 1975), we proposed, based on the observation that the lethal effect of *mle* is restricted to single *X* individuals, that the *mle* gene interacts with a gene(s) on the *X* chromosome which is not dosage-compensated. A gynandromorph analysis, which is being planned, should provide information on this hypothesis. The analysis coupled with detailed histological examination might also provide information with respect to the primary site (focus) of the *mle* action on whether it is a larval tissue, an imaginal external (histoblast is still a possibility), or an imaginal internal (HOTTA and BENZER 1973; BRYANT and ZORNETZER 1973; KANKEL and HALL 1976).

Mutants are known in *D. melanogaster* (LINDSLEY and GRELL 1968) that affect each of the morphogenetic abnormalities described in the present study (e.g., Abnormal abdomen, lethal (2) cryptocephal). On the other hand, many late lethals are pleiotropic in their effects and some are defective in the hormonal system (e.g., lethal(2) giant larva). Although we are not yet in a position to

present any solid explanation for the primary action of the *mle* gene, one possibility may be mentioned here since, even if it proves not to be the case in the *mle* gene, it may still be pertinent in our attempt to investigate the general problem of sex differentiation.

It may be reasonable to assume that there are some hormones such that the level is high in the female and low in the male. A difference in hormonal titer between the two sexes is suggested in Drosophila, for the imaginal disc cells transplanted into the adult body cavity proliferate more effectively when the host is a female rather than a male (cf. OBERLANDER 1972). Although the primary sex differentiation in Drosophila is not affected by hormones, some hormones are known to have effects on the secondary sex differentiation such as spermatogenesis and ovary maturation (cf. DOANE 1973). It seems reasonable to assume further that some genes responsible for the hormone titer are on the *X* chromosome and are not dosage-compensated. The imaginal discs of *mle/mle* males apparently have a normal competence to the hypothetical hormone level in normal males, since these discs differentiated into adult structures when implanted into normal male larvae. Thus the *mle* gene probably does not affect the cell's affinity to bind hormones or the number of binding site, the process presently under active investigation by several workers (YUND and FRISTROM 1975). Instead, the mutant may interact with the hypothetical genes on the *X* chromosome to lower the hormonal level in the single-*X* flies below the threshold. Since various imaginal discs have different levels of competence to the metamorphosis hormones (cf. OBERLANDER 1972), the lowered hormone titer as hypothesized here may account for the morphogenetic abnormalities observed in the present study. Inadequate or unsustained supply of hormones, probably also causing the abnormalities in the development of internal organs, may explain the lethality in the single-*X* individuals.

If the above hypothesis holds then the following statements will emerge: (1) A class of sex-specific or sex-differential lethals may be composed of the genes responsible for the production or regulation of activity of hormones. (2) These genes are not involved in the process of primary sex differentiation and thus no interactions with the sex-transforming genes will be observed. (3) The lethal stage may occur at any time in the life cycle depending on the specific developmental process affected by the hormone.

Although the causal analysis of biochemical differences between two sexes may be a quite complicated one, it may be hoped that the analysis of sex-specific lethals with lethal phase at various developmental stages will provide a first step toward the understanding of mechanisms of such systems. Attempts to obtain more sex-specific lethals and to characterize their effects are underway in our laboratory. There is some reason to believe that the sex-specific lethals are not so limited in number, since they have been recovered from nature, spontaneously from laboratory stocks, and from a laboratory stock by mutagenesis (cf. FUKUNAGA, TANAKA and OISHI 1975), and all of them so far known are nonallelic. We already have another *mle*-like mutant which is presently under investigation.

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