MUTAGENESIS AT A COMPLEX LOCUS IN DROSOPHILA WITH THE MONOFUNCTIONAL ALKYLATING AGENT, ETHYL METHANESULFONATE

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Received May 25, 1967

THE use of chemical mutagens in Drosophila usually results in the production of mutants which are composed of mutant and nonmutant tissue. Mutants of this type are referred to as mosaic or fractional, as opposed to the complete or whole body mutants so commonly found in studies of X-ray mutagenesis. For the detection of mosaic and complete mutants, as well as for general mutagenesis studies, the most frequently used genetic system is the sex-linked lethal system. The use of this system has, however, certain disadvantages, especially for the detection of mosaics when using a powerful mutagen such as ethyl methanesulfonate (EMS). One reason for this is the misclassification of double mosaics as completes; these "pseudocompletes" arise from a two-hit event occurring at different sites along the two strands or subunits of a chromosome with one hit on each strand (EPLER 1966; JENKINS 1967). Another problem arises when more than one lethal hit occurs along the same strand or subunit of a chromosome. Such multiple-hit mutants would be classified as single-hit events, and they would result in an underestimation of the frequency of induced lethal mutations.

Using a complex locus, such as dumpy, to study chemical mutagenesis has some advantages. If an intragenic double bilateral or complex mosaic were induced, two types of dumpy mutants would be produced—both detectable under most circumstances. This would permit a more accurate determination of the number of mutations arising at a given locus as well as the relative mutability of the various mutant sites within that locus.

The dumpy locus, with its multiplicity of mutant sites and wide range of phenotypes, is an ideal locus for studying genetic fine structure and mutagenesis. The dumpy phenotype involves the thorax and its derivatives, the legs and wings, as well as an embryonic lethal effect. A mosaic dumpy mutant would be detected three times more frequently than would a mosaic eye mutant because of the larger number of cleavage nuclei which participate in the formation of thoracic tissue; however, probably only 75% of the dumpy mosaics induced are actually detected in the somatic or germinal tissues because of the presence of mutant tissue in nondetectable areas such as the head or abdomen (LEE, KIRBY, and DEBNEY 1967). Nevertheless, compared to the sex-linked lethal system, dumpy is a preferable system to use because both the soma and the gonad can be analyzed for

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mutant tissue, whereas in the sex-linked lethal system only the gonad can be analyzed. A mutant, such as yellow body, that invests nearly 100% of the surface tissue with a distinguishable phenotype, would be more conducive to studies of mosaicism, but it would not lend itself as well to a structural analysis because it lacks the pleiotropism of complex loci.

Three mutagens, other than EMS, have been employed in the study of the dumpy locus: nitrosomethylurea (CORWIN 1966), ICR-170 (formerly referred to as ICR-100—CARLSON and OSTER 1962; SOUTHIN 1966), and X rays (CARLSON and SOUTHIN 1962). EMS, nitrosomethylurea (NMU), and ICR-170 are alkylating agents, but their reaction mechanisms probably differ. NMU probably methylates the nucleic acid directly and also indirectly through an NMU breakdown product, diazomethane (LOVELESS 1966); EMS directly ethylates the nucleic acid, inducing primarily transition mutations (KRIEG 1963; LAWLEY and BROOKES 1963); ICR-170 probably behaves as an acridine by inducing frameshift mutations (CARLSON, SEDEROFF, and COGAN 1967; SEDEROFF 1966). On theoretical grounds, differences would be expected in the mutagenesis and/or mosaicism patterns for the various agents employed, owing to their structural diversity and reaction differences.

In this report, EMS was used to study the frequency of mutation at the dumpy locus and the characteristics of dumpy mosaicism. Its capacity for inducing mutations in Drosophila has been well established (FAHMY and FAHMY 1957; ALDERSON 1965; EPLER 1966; JENKINS 1967), but a detailed analysis of its actions on a complex locus, such as dumpy, has never been attempted. Its effects will be compared to the effects of the other three mutagens.

MATERIALS AND METHODS

Stocks: Two stocks were employed: Oregon-R wild type, and *ed ov cl* (*ed* = echinoid eyes, 2, 11.); *ov* = a dumpy pseudoallele characterized by truncated wings (o) and thoracic vortices (v), 2, 13; *cl* = clot eyes, 2, 16.5).

Mutagen: EMS was administered to the wild-type males in a 0.7% acetate buffer solution of pH 6.8. It was either fed or injected into the 3-day old males. For injection, a glass needle was prepared by drawing out a pasteur pipette over a microburner. The mutagen was injected into the ventral abdomen. For feeding, the males were placed in a vial containing only a piece of Kimwipe paper tissue saturated with the buffered EMS at a glucose molarity of 0.1 (E. B. LEWIS, personal communication). The males were left on this medium for 24 hours. With the techniques employed in this study, it was not possible to quantitate the amount of mutagen effectively administered for either technique. For a discussion of the injection procedure, see CARLSON and OSTER (1962).

Mating procedure: The treated males were allowed 24 hours to recover from the EMS treatment, although at the doses administered the compound did not seem to affect the flies adversely. Each male was place in a vial, containing the standard cornmeal-agar-molasses medium seeded with live yeast, with three virgin *ed ov cl* females. The males were transferred to fresh virgin females every two days, each transfer constituting a brood. This type of brood analysis allows the observer to sample premeiotic, meiotic, and postmeiotic sperm cells. The first three broods (0 to 7 days) sample postmeiotic cells, the fourth brood samples primarily meiotic cells, and the last three broods represent samples of premeiotic cells. Such a timetable of spermatogenic events has been established in previous experimentation (CHANDLEY and BATEMAN 1962).

Dumpy locus: Three phenotypic expressions characterize the dumpy locus: (1) truncated

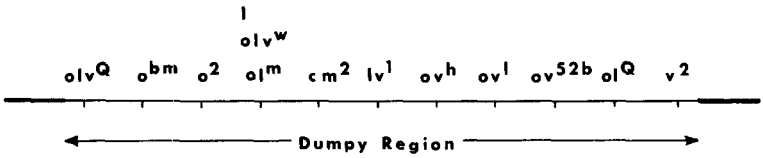

FIGURE 1.—The sequential arrangement of the dumpy mutant sites within the dumpy locus. The spacings do not represent actual map-unit distances (after GRACE 1966).

wings (o), (2) thoracic vortices (v), and (3) an embryonic lethal effect (l). The pseudoalleles of this gene may express any one of these phenotypes either singly (*o, v, l*), or in combination with one another (*ov, ol, lv, olv*). There is also a comma mutant (*cm*) characterized by comma-like indentations at the anterior thorax. Associated with the *ol* and *olv* mutants is a stubby-legged effect. The sequential arrangement of the intragenic pseudoallelic sites is shown in Figure 1 (GRACE 1966).

There is some intragenic complementation among these pseudoalleles in the *trans* configuration—for example, *ov/l* is wild-type, *ov/o* expresses truncated wings, and o^2v^2/l is semilethal but wild-type phenotypically. In general, compounds of dumpy pseudoalleles only manifest the traits they have in common. For a more comprehensive description of the dumpy locus, see CARLSON (1959).

Scoring and classification procedure: The F_1 flies were scored for visible dumpy mutations and they were classified as mosaics or completes. Each of the induced mutants in the F_1 was crossed to three *ed ov cl* flies and the F_2 progeny were examined for the transmission of the mutation from the F_1 mutant.

The classification of the dumpy mutants induced with EMS is based primarily on the visible phenotype. The mutant *ol* is distinguished from *o* by the presence, in *ol*, of a more extreme wing effect and a reduced leg and body effect. The mutant *olv* differs from *ov*, having a more extreme wing effect as well as a more severely affected thorax, in addition to the reduced leg and body size. The mutant *lv* is distinguished from *v* by the presence, in *lv*, of a pronounced comma effect. The reliability of such a classification procedure was borne out by randomly selecting 12 transmitting EMS-induced dumpy mutants (4 *olv*, 4 *lv*, 3 *ol*, 1 *o*) and balancing them over *Cy Stw L*. Tests were carried out on each mutant to determine lethality and all the mutants were found to be exactly as originally classified. Additional evidence regarding the reliability of this classification comes from the data which show that 77% of the transmitting mutants were correctly classified in the F_1.

An additional class of mutants, bearing normal phenotypes but mutant gonadal tissue, was also assayed. Such "cryptic mutants" were predicted and found in ICR-170 studies (CARLSON 1964; SOUTHIN 1966). The frequency of cryptic mutation was calculated by randomly selecting phenotypically nonmutant F_1 males and virgin females and crossing them to *ed ov cl* flies. If F_2 dumpy progeny were produced, the F_1 parent fly was said to be gonadally mutant-somatically nonmutant (cryptic).

RESULTS

Ethyl methanesulfonate is a potent mutagenic agent, inducing mutations at the dumpy locus with a frequency at least as high as 2%. This value is obtained when the data from the F_1 visible mutations are combined with the data from the cryptic analysis (Tables 1, 2, 3). EMS induces mutations at the dumpy locus proportional to the molarity of the EMS injected (JENKINS 1967). A higher mutation frequency was obtained when EMS was fed than when it was injected (Table 2). Other differences, such as percent transmissibility, percent mosaicism, and mutational spectrum, were statistically nonsignificant. Further remarks will,

TABLE 1

Mutation frequency at the dumpy locus using various concentrations of ethyl methanesulfonate injected into the ventral abdomen

EMS Concentration	No. flies scored	No. dumpy mutants	F_1 frequency	No. transmitted dumpy mutants	No. non-transmitted dumpy mutants	No. doubted*
0.005M	24972	28	0.11%	8	18	2
0.01M	11400	24	0.21%	9	10	5
0.02M	23711	116	0.49%	27	75	14
0.04M	7131	46	0.65%	12	22	12
Control†	13789	3	0.02%	2	1	0

* Mutant died before transmission determined.
† Injected with buffer.

TABLE 2

Comparison of the mutagenicity at the dumpy locus when 0.02M ethyl methanesulfonate was fed and when it was injected

Technique	No. flies scored	No. dumpy mutants	F_1 frequency	No. transmitted dumpy mutants	No. non-transmitted dumpy mutants	No. doubted*
fed	16611	191	1.15%	58	87	46
injected	23711	116	0.49%	27	75	14

* Mutant died before transmission determined

TABLE 3

Analysis of the frequency of inducing cryptic mutants in the F_1 (F_1 mutants that are somatically nonmutant — gonadally mutant)

EMS concentration and method of administration	No. of F_1 progeny analyzed	No. of F_1 phenotypic dumpy mutants	Frequency of inducing F_1 phenotypic mutants	No. of F_1 phenotypic wild-type progeny used	No. of F_1 phenotypic wild-type progeny producing dumpy F_2	Cryptic frequency
0.02M fed 24 hr	1406	30	0.0213	746	12	0.0161
0.03M fed 12 hr	2339	36	0.0154	733	2	0.0027
0.06M injected	1586	37	0.0233	218	3	0.0138

therefore, refer to a consolidation of the feeding and injection experimental data.

The most striking aspect of EMS mutagenesis is its exclusive action on postmeiotic sperm cells (Table 4). Using fiducial limits at a 5% level of significance, no increase over spontaneous mutation frequency was observed in the premeiotic cells (STEVENS 1942), the spontaneous mutation frequency for dumpy being 2.0×10^{-4} to 3.3×10^{-4}. A similar pattern of meiotic and premeiotic cell resistance was found using methyl methanesulfonate, where 87% of all the sex-linked lethal mutations induced were in the postmeiotic cells (BATEMAN and CHANDLEY 1964). The resistance to EMS might be attributed to a cytoplasmic barrier. Support for this hypothesis comes from data which shows that in reciprocal crosses involving EMS-fed or EMS-injected females (wild-type) and *ed ov cl* males, the mutation frequency was much lower than in the reciprocal cross (24/12119 compared to 83/6424).

Thirty-five percent of the F_1 phenotypic dumpy mutants tested were also found to be gonadally mutant, when the progeny of these mutants were examined in the F_2 (Tables 1, 2). This value is comparable to that obtained with NMU and ICR-170 (25 to 28%) by CARLSON and OSTER (1962), SOUTHIN (1966), and CORWIN (1966). The higher transmission frequency of 72% for X-ray induced mutations is probably due to the difference in the mode of action of X rays compared to chemicals, X rays resulting in more whole body or complete mutations whose transmissibility is close to 100% (CARLSON and SOUTHIN 1962).

The phenotypic spectra of the F_1 dumpy mutants (Table 5) and the transmitted dumpy mutants (Table 6) are not dissimilar from the spectra obtained for NMU (CORWIN 1966), ICR-170 (CARLSON and OSTER 1962; SOUTHIN 1966), and X rays (CARLSON 1959 and personal communication; CARLSON and SOUTHIN 1962). Most of the mutants were *olv* and *lv* (58 to 78%), while the remainder—

TABLE 4

Analysis of the frequency of inducing phenotypically complete and phenotypically mosaic dumpy mutants during the stages of spermatogenesis. Each brood represents a 2-day interval

	Brood						
	postmeiotic			meiotic		premeiotic	
	1	2	3	4	5	6	7
EMS:							
F_1 flies scored	22388	18021	9081	8384	8261	10882	4808
Complete mutants	34	39	12	0	2	3	0
Mosaic mutants	120	108	67	13	4	3	0
Total	154	147	79	13	6	6	0
Frequency (%)	0.68	0.81	0.86	0.15	0.07	0.06	0.00
Control:							
F_1 flies scored	2551	2184	1623	1420	2011	1993	2007
Complete mutants	0	0	1	1	0	0	0
Mosaic mutants	1	0	0	0	0	0	0
Total	1	0	1	1	0	0	0
Frequency (%)	0.04	..	0.06	0.07	..	..	..

TABLE 5

Classification of the F_1 EMS-induced dumpy phenotypes, as they appeared in the F_1

	Dumpy phenotype							
	olv	o	ol	lv	ov	v	cm	Total
EMS:								
Complete	30	5	15	33	3	0	4	90
Mosaic	102	44	45	70	26	21	7	315
Total	132	49	60	103	29	21	11	405
CONTROL:								
Complete	0	0	0	2	0	0	0	2
Mosaic	1	0	0	0	0	0	0	1
Total	1	0	0	2	0	0	0	3

Data include those mutants which did not transmit and those that died before transmission could be determined.

ol, *o*, *ov*, *v*, and *cm*—accounted for 22 to 42%. Although a wide range of mutagens has been employed to investigate the mutagenicity of the dumpy pseudoalleles, the spectra of the induced mutants remain fairly constant. Sufficient data has not been collected to properly analyze the spectrum for spontaneous dumpy mutants.

Another aspect of mutant transmissability may be called "phenotypic fidelity". Phenotypic fidelity may be defined as the frequency with which a given class of F_1 dumpy mutants (such as *olv* or *ol*), classified according to their phenotypes in the F_1, transmits to its progeny the genotype which will give rise to the same F_1 phenotype in the F_2. For example, if 20 transmitting dumpy mutants, classified in the F_1 as *ol*, transmit as 15 *ol* and 5 *olv*, then the phenotypic fidelity would be 0.75. In only 23% of the cases of transmitted mutations was there a disagreement in the classification of the phenotype found in the F_2 compared to that of the F_1. In virtually every such instance of disagreement, the phenotype of the F_1 was less severe than that of the F_2 (Table 7). A possible explanation for this 23% discrepancy would involve the particular patterns of mosaicism characteristic for

TABLE 6

Classification of the transmitted *EMS-induced dumpy mutants and their comparison with ICR-170, X-ray, and nitrosomethylurea-induced dumpy mutants*

	Dumpy mutant induced						
Mutagen	*olv*	*o*	*ol*	*lv*	*ov*	*v*	*cm*
EMS	96	9	30	77	7	4	0
ICR-170*	72	5	23	48	1	1	0
X rays*†	110	4	34	84	4	0	0
NMU‡	32	1	10	19	0	1	0
Spontaneous§	0	0	1	3	0	0	0

* E. A. CARLSON (personal communication).
† CARLSON (1959); CARLSON and SOUTHIN (1962).
‡ CORWIN (1966).
§ A. W. SHERMOEN (personal communication).

TABLE 7

"Phenotypic Fidelity". A tabulation of the genotypes transmitted to the F_2 by the phenotypes of the F_1

F_1 phenotype	F_2 genotype							Total	Phenotypic fidelity
	o	*olv*	*ol*	*cm*	*lv*	*ov*	*v*		
o	6	5	6	0	0	1	0	18	0.35
olv	0	66	0	0	0	0	0	66	1.00
ol	1	5	21	0	0	0	0	27	0.76
cm	0	0	0	0	4	0	0	4	0.00
lv	1	1	0	0	57	0	1	60	0.94
ov	0	11	0	0	0	6	0	17	0.33
v	0	3	0	0	9	0	2	14	0.09
+	1	5	3	0	7	0	1	17	...

the F_1. A distribution of nonmutant tissue interlacing mutant issue would modify a mutant phenotype, making it less extreme. Some of the transmissions may actually be examples of double mosaics—for example, an F_1 *lv* that transmitted as an *o* may have had an *lv* in the soma and an *o* in the gonad. These would be complex or fore-and-aft double mosaics in contrast to the more familiar double bilateral mosaics which manifest both allelic phenotypes in the soma (CARLSON and SOUTHIN 1962).

Complete mutations were rare in this study. Of the 405 induced mutants in the F_1, 77% were phenotypic mosaics. This value increases to 94% if the phenotypic complete mutants that were gonadally mosaic or nonmutant are reclassified as mosaics. A further increase in mosaics is obtained when the data for the cryptic mutants are included in the calculations (Table 3). Cryptic mutants are phenotypic nonmutants that carry mutant tissue in all or part of their gonads, and are, therefore, mosaics. EMS induced cryptic mutations at about 50% the rate that it had induced F_1 phenotypic visible mutations. Making use of this fact, and recalculating the frequency of mosaicism, a value of 98% is obtained. It is likely that this value could be further increased by adding that proportion of the F_1 phenotypically complete - gonadally complete mutants carrying non-dumpy tissue in a nonanalyzable part of the soma, and reciprocally, those F_1 phenotypically nonmutant - gonadally nonmutant individuals carrying dumpy in a nonanalyzable segment of the soma. Theoretically, such classes should exist.

An estimate of the average amount of mutant and nonmutant tissue in the gonads can be determined. Using data complied from all the gonadally mosaic males producing ten or more progeny, 416/740 F_2 progeny were dumpy; this demonstrates that 56% of the gonadal tissue contained mutant cells.

DISCUSSION

The mechanism by which EMS induces mutations in Drosophila is not known, but the mechanisms proposed for the bacteriophage T4 (KRIEG 1963) could be extrapolated to Drosophila. In T4, there are two possible mechanisms for EMS

mutagenesis: (1) the ethylation of guanine or adenine and the subsequent mispairing to give rise to transition mutations; (2) hydrolysis of the ethylated bases from the DNA molecule and the subsequent errors of incorporation at these gaps. Either one, or both of these mechanisms may account for EMS-induced mutations in Drosophila, but a third possibility cannot be discounted: an ethylated intermediate compound, such as an ethylated amino acid or protein, may possess mutagenic properties.

In T4 and Neurospora, EMS seems to have a delayed effect (GREEN and KRIEG 1961; NASIM and AUERBACH 1967). There is a reaction between the EMS and the genetic material, but the mutation may not be fixed for one or more replications. If such a delayed effect existed in Drosophila, it would be expected that a large fraction of the mutants induced in premeiotic broods, as well as those in postmeiotic broods, should be mosaic. However, in this work it was shown that EMS had virtually no effect on premeiotic cells, and therefore no valid test can be made for an EMS-induced delayed effect in Drosophila with the techniques used here.

There is no convincing evidence in this study to suggest that EMS induced breakage events. Only one mutant was found to be the result of a chromosome break—an *ov* mutant showing a variegated position effect. Cytologically, it involved a translocation of the distal portion of the 2L chromosome terminating at the dumpy region between bands 25A1–2 and 25A3–4, and the entire right arm of the third chromosome (R. HENDRICKSON, personal communication).

The mutagenic effects of four mutagens studied at the dumpy locus and their spectra show remarkable similarity, regardless of their diverse effects on the cell's genetic material. This suggests that an alteration in a specific area of the dumpy region, regardless of its origin, will result in a specific phenotype. If so, it could suggest that the size of portions of the dumpy region giving rise to lethal phenotypes are larger than those giving rise to a nonlethal phenotype. Based on this possibility, one could construct a model of the dumpy region based on relative mutation frequencies rather than on mutant sites (Figure 2). The areas within the dumpy region would be based on the proportions of the transmitted dumpy pseudoalleles (Table 6).

This model doesn't eliminate the possibility that nonlethal dumpy mutations may arise through a frame-shift type mechanism. Indeed, they probably have. It

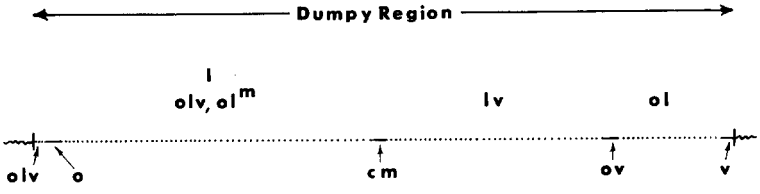

FIGURE 2.—Model of the dumpy region based on frequencies of mutation rather than mutant sites (frequencies of the transmitting mutants were used—see Table 6). $olv = 0.43$; $lv = 0.35$; $ol = 0.13$; $o = 0.04$; $ov = 0.03$; $v = 0.02$. The *l* and *cm* frequencies were not known, but since their sites were determined, they were given minimal area.

was stated previously that ICR-170 induced only lethal dumpy mutations and that this supported an acridine mutagenic mechanism (CARLSON, SEDEROFF and COGAN 1967). The nonlethal dumpy mutations that appeared were assumed to have arisen spontaneously. With the model shown, one would predict that nonlethals would be induced with ICR-170 but their frequency would be much lower than the frequency of EMS-induced nonlethals. This difference is attributed to the larger portion of the DNA involved in a frame-shift mechanism compared to the single base-pair change induced by EMS. The fact that only 4% of the ICR-170-induced mutations were nonlethal compared to 9% for EMS lends some support to this idea.

To date, eight types of dumpy mutants have been found and studied, but a ninth allelic phenotype, a "stubby-legged" mutant, may be recovered in the future. This is an inference based on two observations: (1) stubby-legged mutants have been found and one case transmitted this phenotype (E. A. CARLSON, personal communication). Unfortunately, this stock was lost before it could be studied past the F_2 generation; (2) a stubby-legged effect is always associated with the *ol* and *olv* pseudoalleles of dumpy.

The ability of EMS to induce almost 98% mosaics in postmeiotic cells is one of its most striking qualities. The origin of these mosaics is readily interpreted by the Watson-Crick model of complementary DNA strands and semiconservative replication. The origin of the 2% completes (if they do exist as true complete mutants) is more difficult to explain. NASIM and AUERBACH (1967) propose that these completes arise through a repair mechanism, in which a mismatched base pair is deleted and a matching base pair is inserted; as an alternative, they suggest a dual mechanism, such as the cross-linking of complementary strands, which is essentially different from the mechanism giving rise to mosaics.

When the composition of the mosaic gonads was determined, it was found that 56% of the tissue was mutant. This compares favorably with values of 54 and 48% respectively found in the work of ALDERSON (1965) and EPLER (1966) with EMS. In studies with ICR-170, it was found that 44% of the gonad was mutant (SOUTHIN 1966; CARLSON and SOUTHIN 1963). The values, however, may be misleading. They all approximate 50%, but in actuality, very few individuals had 50% mutant tissue in their gonads. There was a wide variation from 2 to 96% and the frequency distribution was essentially a straight line, parallel to the X-axis (Figure 3). This indicates that there is a poorly mixed sample of cleavage nuclei selected for the germ cell line.

The author wishes to express his appreciation to DR. ELOF A. CARLSON, DR. RONALD SEDEROFF, and MR. DALE GRACE for their stimulating discussions and criticisms and to DR. ROBERT J. HENDRICKSON for his cytological analysis of a variegated dumpy mutant. MR. ANTONY W. SHERMOEN was kind enough to allow me to use some of his unpublished data, and MR. STEPHEN HANDLER provided valuable technical assistance. This work was supported in part by a National Science Foundation grant to DR. ELOF A. CARLSON.

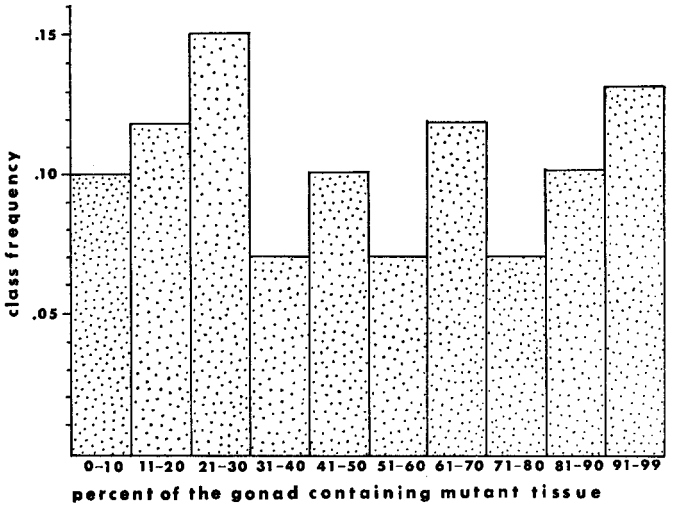

FIGURE 3.—Frequency distribution of the percent mutant tissue in the gonads of 61 gonadally mosaic males. Some of the data come from ICR-170 studies (SOUTHIN 1966), and the remainder from EMS studies.

SUMMARY

Ethyl methanesulfonate (EMS) is a potent mutagenic agent in Drosophila, inducing mutations at the dumpy locus with a frequency of at least 2%. Mutations were found in the progeny of males, both after feeding and after injection with EMS, and the induced mutation frequency was proportional to the concentration injected. EMS acted primarily on postmeiotic sperm cells, the premeiotic and meiotic cells remaining relatively immune to its effects. Ninety-eight percent of all the induced mutants detected in the F_1 were mosaic (containing mutant and nonmutant tissue). Thirty-five percent of all the F_1 mutants contained mutant tissue in their gonads. The average amount of mutant tissue in a mosaic gonad was 56%, similar to values found in studies with ICR-170 and NMU and in other studies with EMS. However, these values, all around 50%, may be misleading because the variances are so large.—With only 23% of transmitted mutations was there a disagreement in the classification of the phenotype found in F_2 compared to F_1. In virtually every such instance of disagreement, the phenotype was less severe in F_1 than in F_2.—A comparison is made of the transmitting dumpy mutants induced with EMS, nitrosomethylurea, X rays, and ICR-170, and a model of the dumpy region is constructed based on this comparison.

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