Mutagenesis at Five Pteridine Pathway Loci in *Drosophila melanogaster*: Genetic and Biochemical Characterization

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Summary

In order to carry out a genetic and biochemical analysis of pteridin pathway eye colour loci, sixteen strains of five eye colour mutants of *Drosophila melanogaster* (2 dke, 7 sf, 3 se, 3 Hn^r and 1 bw) from natural populations were used. Four EMS mutagenesis experiments were carried out to produce induced mutants of the same loci. 54 mutants (mosaics and completes) were obtained but only 7 (4 sf, 1 bw, 1 Hn^r and 1 dke) could be isolated. The 40.48% of the mutations were mosaics. The percentage of mutants appeared during the four first days (85.19%) was significantly higher to the percentage of mutants appeared during the four following (14.18%). Viabilities of EMS-induced mutants were similar to that of the natural ones. For the induced mutants viability at 25°C was higher than at 16°C and heterozygotes had a higher viability than mutant homozygotes. The low mutagenesis frequencies and the lack of some metabolites for Hn^r and se mutants suggest an important role of these mutants in the pteridine pathway.

Key words: Mutagenesis, Eye color mutants, Pteridines, *Drosophila melanogaster*

Introduction

Two classes of pigments contribute to the final colour of *Drosophila* eyes: the ommochromes and the pteridines. The ommochromes pathway was established long time ago, however, the pathways for the biosynthesis of the red pigments still remain incomplete at present. A hypothetical pathway (Fig. 1), was proposed (1). The only loci which are sure involved as structural genes in this pathway are Punch (GTP cyclohydrolase enzyme), purple (sepiapterine sintase A enzyme) and rosy (xanthin dehydrogenase enzyme). The study of eye colour mutants in *Drosophila* means an advance in the knowledge of the pathway, nevertheless the location of structural genes is difficult principally due to pleiotropic effects of genes and sometimes to lethality. In the present work, five pteridine pathway mutants were used: 3 of them located in the second chromosome

of *Drosophila melanogaster* (dark-eye, safranin and brown) and the other two located in the third one (Henna-recessive and sepia). The function of these five mutants in the pathway is unknown but Ferré *et al.* (2) proposed the hypothesis that Hn^r blocked the formation of tetrahydropterin and se the formation of acetyldihydrohomopterin (Fig. 1). Environmental mutagenesis experiments were carried out in order to get induced mutants. The Ethyl methanesulfonate (EMS) was chosen among all the chemical mutagens because its water solubility, stability effectiveness and easy handling (3-6). A genetical and biochemical study of natural and induced mutants of the five loci was made in order to contribute to understand the role of these mutants in the pteridin pathway.

Materials and Methods

Drosophila strains and culture

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Figure 1. Proposed pathway for pteridine biosynthesis in *Drosophila melanogaster*.

Sixteen strains of five eye colour mutants of *Drosophila melanogaster* (2 dke, 7 sf, 3 se, 3 Hn^r and 1 bw) from natural populations were used. These strains were obtained by Nájera and Ménsua (7) from a total of six captures from three different habitats; a cellar in Requena (Valencia, Spain) a vineyard in El Portón (4 km away from the cellar) and a semi built up pinewood at La Cañada (70 km away from Requena) on spring and autumn in 1981. Marker strains used to obtain EMS-induced mutants were al b cn sp homozygous males for the 2nd chromosome and st ri ss homozygous for the 3rd one; also, standard mutant females for each one of the five eye colour mutations were used to be crossed by the mutagenized males except for bw. For this locus the deficient strain Df(2R)bw⁵, bw⁵/In(2L)Cy, dp^{iv2} was used. The strains were obtained from the collection in Bowling Green (USA) except st ri ss which was set up by introducing the st mutant in the ri ss strain. All strains were maintained at 25±1°C, 60±10% relative humidity under constant lighting, in 125 ml bottles with 30 ml of a standard medium of corn flour, agar-agar, sugar and liver yeast.

Mutagenesis

Four mutagenesis experiments were carried out by feeding EMS to *Drosophila melanogaster* males, homozygous for second chromosomes marked with al b cn sp or for third marked with st ri ss. Feeding method was chosen because its power in producing punctual mutations without affecting development is higher than inhalation or injection methods. Only males were fed because of the higher mutagenesis action of EMS on spermatocytes than on oocytes.

So, three day-old males were fed in 40 mM EMS solution in 5% sucrose for 24 hours at room temperature (8). The mutagenized males were allowed 24 hours to recover. Each male was placed in a vial containing standard medium and crossed 6/8 virgin mutant females, homozygous for the standard allele of one of the five studied mutations. Males were transferred to fresh virgin females, every two days, each transfer constituting a brood. This type of brood analysis allows to sample premeiotic, meiotic and postmeiotic sperm cells. The first three broods (0 to 6 days) sample postmeiotic sperm cells, the fourth brood samples primarily meiotic cells and the last broods sample premeiotic cells. The induced mutants were isolated from mass F₁ cultures according to the following scheme:

where * = one of the five standard mutants (sf, se, bw, Hn^r, dke) 1, 2, 3, 4 = marker mutations. By inbreeding, individuals with homozygous mutant phenotype

were selected from the progeny and the EMS-induced strain was set up.

Complementation analysis

Reciprocal crosses of all pairwise combinations of natural and induced mutants were carried out at 25°C and 800/1000 progeny were scored.

Viability and developmental time

For viability testing flies were left for a maximum of 12 hours in the usual medium (9) and afterwards the eggs were collected. One hundred eggs were placed in each vial and ten repetitions were made for each case. The number of emerged adults was counted. For developmental time testing, the formula ($\sum_i n_i \times i / n_i$) was used.

where n_i = total flies emerged in day i
 n_t = total flies emerged in the vial.

A total of ten repetitions were also made in each case. Viability and developmental time of EMS-induced mutants were analysed at two different temperatures, 25° and 16°C. These parameters were also studied in all the heterozygous combinations of the induced mutants with the wild strain Or-R.

Biochemical procedures

The procedure of Ferré *et al.* (2) was used to obtain the chromatographic pattern of each EMS-induced mutant with a modification: the preparation of the samples consisted of homogenizing 20 female heads, 15 male heads and 5 male bodies together. Quantitative estimation of the red eye pigments from the induced mutants was carried out following the procedure of Real *et al.* (10) and quantitative estimation of isoxanthopterin in these mutants was carried out according the method of Silva and Ménsua (11). In order to improve visual resolution of pteridine levels, we removed ommochrome pigments from eye by including the *cn* or *st* mutations in the marker strains.

Results

Toxicity test

Assuming the importance of LC50 (Lethal concentration for 50% of the individuals) in order to know the equilibrium between mutagenicity and mortality, toxicity curves were made and a LC50 of 36.43 mM was obtained. So, 40 mM EMS solution was used since at lower concentrations the frequency of induced mutations is under the sensitivity of the test.

Mutagenesis experiments

Four mutagenesis experiments were carried out at this EMS concentration. The number of total F_1 flies screened for each mutation (grouping the four experiments together), the number of mosaics obtained for each strain, its frequency, the number of total induced mutants and the number of recovered from them, their frequencies and the total mutation rates for each one of the five loci are shown in Table 1. In total, 54 mutants (mosaics and completes) were obtained but only 7 could be isolated. 4 of them were safranin (*sf2MN*, *sf4MN*, *sf5MN* and *sf6MN*), 1 Henna-recessive (*Hn^r3MN*), 1 dark-eye (*dke3MN*) and 1 brown (*bw2MN*). Considering these five loci together, the 40, 48% of these mutations were mosaics. Figure 2 shows the order of appearance of the different mutants through the time. The percentage of mutants appeared during the four first days (85.19%) is significantly higher to the percentage of mutants appeared during the four following (14, 18%) (Table 2). This shows that EMS mutagenesis has exclusive action on postmeiotic sperm cells. No cryptic mutants were found for neither of the five mutations.

Complementation test

The induced mutants were crossed with the natu-

Figure 2. Order of appearance of the induced mutants through the time.

Table 1. Mosaic, complete and total frequencies for each one of the five mutants

Mutation	n° flies screened	Mosaics		Completes				Total	
		n°	freq.	n°	freq.	recov.	freq.	n°	freq.
se	32198	1	3.1×10^{-5}	0	0	0	0	1	3.1×10^{-5}
sf	19654	2	1.1×10^{-4}	8	4.1×10^{-4}	4	2.0×10^{-4}	10	5.1×10^{-4}
bw	18654	4	2.1×10^{-4}	9	4.8×10^{-4}	1	5.3×10^{-5}	13	6.9×10^{-4}
Hn ^r	39947	5	1.3×10^{-4}	7	1.8×10^{-4}	1	2.5×10^{-5}	12	3.0×10^{-4}
dke	31598	7	2.2×10^{-4}	11	3.5×10^{-4}	1	3.2×10^{-4}	18	5.7×10^{-4}

freq (frequency); recov (recovered)

Table 2. Percentage of mutations appeared during the first 4 days versus the subsequent 4

Mutation	First 4 days			Subsequent 4 days		
	mos	com	tot	mos	com	tot
sf	100.00	87.50	90.00	0	12.50	10.00
dke	100.00	81.82	88.89	0	18.18	11.11
bw	75.00	100.00	92.31	25.00	0	7.69
se	100.00	0	100.00	0	0	0
Hn ^r	40.00	85.71	66.67	60.00	14.29	33.33
Total	78.95	88.57	85.19	21.05	11.43	14.81

mos (mosaics); com (complete mutants); tot (totals: mosaics + completes)

ral strains (with the same mutation) and the four induced mutants for sf also among them. In all cases no complementation in the production of the eye colour phenotype has been seen.

Viability and Developmental Time

An analysis of variance (ANOVA) of the mean viabilities for all mutant strains (natural and induced) was made for each one of the five mutations. The means that do not differ significantly were grouped by underlined (Table 3). Viabilities of EMS-induced mutants are similar to that of the natural ones. With regard to the heteroallelic combinations, viability is, in general, higher than viability of the parental strains except for se mutants. A factorial analysis from the sex index indicate variation of viability in relation with the strains and with the sex. In sf, se and bw strains both factors are significant and also its interaction. In Hn^r both factors are significant but not the interaction indicating the higher viability of one sex over the other and in dke there are only significant differences among strains and not between sexes. The viability of the induced mutants was higher at 25° than at 16°C except for bw2MN, although in dke3MN and sf2 MN strains differences were not significant (Table 4). Differences in eye colour among the mutants cultured at these two different temperatures were not observed. In all cases heterozygotes had a higher viability than mutant homozygotes although for sf4 MN strain differences were not significant (Table 4). In general, a high variability and a trend to increase the mean developmental time for the induced mutants was observed. With regard to the heteroallelic genotypes, a trend to decrease the mean developmental time with respect to the parental strains for se heteroalleles and a tendency to increase the mean developmental time with respect to the paren-

Table 3. ANOVA of the mean viabilities of natural and induced mutant strains (Safranin)

Source of variation	df	SS	MS	F
Among groups	10	10.3222	1.0332	77.9162***
Within groups	99	1.3286	0.0134	
Total	109	11.6508		

Mean	Strain
–0.5381	19BO
–0.3740	23BP
–0.3297	51BO
–0.2170	2MN
–0.1171	2PO
0.0675	17VO
0.0839	5MN
0.1574	6MN
0.2962	1VP
0.3853	16VP
0.4046	4MN

(Henna Recessive)

Source of variation	df	SS	MS	F
Among groups	3	9.1080	3.0360	167.8797***
Within groups	36	0.6510	0.0181	
Total	39	9.7590		

Mean	Strain
–0.9028	63BO
–0.7370	28BP
–0.4645	3MN
0.3397	20VP

(Sepia)

Source of variation	df	SS	MS	F
Among groups	2	0.1752	0.0876	4.0697*
Within groups	27	0.5811	0.0215	
Total	29	0.7563		

Mean	Strain
0.1517	3VO
0.2890	85BO
0.3305	33BP

(Dark-Eye)

Source of variation	df	SS	MS	F
Among groups	2	0.8306	0.4153	6.3681**
Within groups	26	1.6956	0.0652	
Total	28	2.5262		

Mean	Strain
–0.2391	58BO
0.0801	3MN
0.1426	1BP

(Brown)

Source of variation	df	SS	MS	F
Among groups	1	0.6553	0.6353	60.0009***
Within groups	18	0.1996	0.0106	
Total	19	0.8259		

df=Degrees of freedom

SS=Sums of squares

MS=Mean squares

F=Values of F test

*=p<0.05 **=p<0.01 ***=p<0.001

C=cellar V=vineyard P=pine-wood A=autumn S=spring

MN=induced mutants

Table 4. Comparison of the viabilities of the induced mutants 1) at two different temperatures (25°C-16°C) and 2) versus its heterozygotes with Or-R

Strain	25°C-16°C		Heterozygote-Homozygote	
	df	t	df	t
sf2MN	18	1.96ns	18	10.02***
sf4MN	18	8.32***	18	1.45ns
sf5MN	18	10.44***	18	6.35***
sf6MN	18	4.25***	18	7.29***
Hn'3MN	18	5.55***	18	25.28***
dke3MN	17	0.19ns	16	6.06***
bw2MN	18	-4.27***	18	25.98***

df=Degrees of freedom

t=Values of t test

***=p<0.01

MN induced mutants

tal strains for sf heteroalleles was observed while the MDT of the heterozygotes is similar to that of the mutant homozygotes.

Biochemical Analyses

The chromatographic pattern of the induced mutants compared to their standards is shown in Table 5. bw2MN and dke3MN have a very different patterns with respect to their standards. sf2MN and sf6MN have the same pattern and sf4MN and sf5MN have a very similar one but different from the other two. Hn'3MN has a decreased level of drosoppterins in respect to the standard with lack of auro-drosoppterin, isoxanthoppterin and pterin. The quantitative estimation of the red pigment (absorbance at 480 nm) and its relative percentage with respect to the wild strain Or-R, and respect to these values for the same standard mutants can be observed in Table 6. bw2MN had a level of pteridines higher

Table 5. Semiquantitative estimation of pteridines in eye colour induced mutants of *Drosophila melanogaster*

Strain	NDP	ADP	DP	SP	ADHP	IXP	PTE	BP
Or-R	+	+	+	+	+	+	+	+
bw st	0	0	0	0	0	0	0	0
bw2MN	-1	-1	-2	+	-1	+	0	+3
dke st	-1	+	-1	+	+	+	-1	+
dke3MN	+	-1	-1	-1	+	-1	-1	+1
Hn st	-1	-2	-1	+2	+1	-1	-1	+
Hn'3MN	-2	0	-2	+1	+1	0	0	+1
sf st	+	+	-1	-1	+	+	+	+
sf2MN	+	+	+	-1	+	-1	+	+1
sf4MN	-1	-1	-1	-1	+	-2	+	+1
sf5MN	-1	-1	-1	-1	+	-1	-1	+1
sf6MN	+	+	+	-1	+	-1	+	+1

+: amount approximately equal to Or-R

+1, +2, +3: increasing amounts

-1, -2: decreasing amounts

0: not detected

MN: induced mutants

st: standard mutants

NDP, neodrosoppterin; ADP, aurodrosoppterin; DP, drosoppterin; SP, sepiapterin; ADHP, acetyldihydrohomoppterin; IXP, isoxanthoppterin; PTE, pterin; BP, biopterin

Table 6. Quantitative estimation of the red pigment of eye colour and quantitative estimation of isoxanthoppterin in male bodies in different standard and induced mutants of *Drosophila melanogaster*

Strain	Red pigment		Isoxanthoppterin	
	Absorbance at 480 nm	% with respect to Or-R	Fluorescence	% with respect to OR-R
bw st	0.010±0.001	1.44±0.13	0.9±0.2	1.62±0.06
bw2MN	0.101±0.004	22.52±0.42	49.6±0.1	91.85±0.27
dke st	0.481±0.006	72.92±0.91	38.0±0.3	68.37±0.96
dke3MN	0.230±0.008	51.22±0.63	40.7±0.2	76.32±0.73
Hn st	0.177±0.003	26.82±0.41	27.9±0.4	50.12±0.76
Hn'3MN	0.103±0.004	22.94±0.15	1.0±0.3	1.09±0.04
sf st	0.280±0.007	42.48±1.03	34.5±0.5	62.04±0.87
sf2MN	0.238±0.005	53.01±0.74	45.2±0.4	86.10±0.78
sf4MN	0.183±0.005	40.76±0.98	24.0±0.2	45.71±0.81
sf5MN	0.182±0.007	40.53±0.84	35.7±0.7	67.94±0.91
sf6MN	0.242±0.007	53.90±0.72	31.9±0.2	60.90±0.82

st: standard mutants

MN: induced mutants

than the standard, approximately 20% of the Or-R level. dke3MN and Hn'3MN had a reduced level of pteridines in respect to the standard mutants and in sf there are two alleles (sf2MN and sf6MN) with a level of pteridines similar to the standard and

the other two (sf4MN and sf5MN) a decreased level. The quantitative estimation of the isoxanthopterin in the induced mutants and in the standard ones is shown in Table 6 as well. The most striking is the practically lack of isoxanthopterin in Hn³MN mutant strain compared with the standard. Opposite to this, we found the existence of isoxanthopterin in the induced bw mutant compared with the lack in the bw standard.

Discussion

Assuming that our interest was centralized in specific gene loci located in 2nd and 3th chromosomes the specific locus test in which puntual mutations are detected through the visible phenotype, was used. Other authors with the same purpose used also this method (5-7, 12-15). The mutation frequencies found in this work were similar or slightly lower than that obtained by other authors for other loci (5.1×10^{-3} for dp, (5); 1.09×10^{-3} for cn, (6); 3.7×10^{-4} for Pu, (12). The frequencies of isolation of the mutants were also lower than those obtained by the previously mentioned authors. Most of the obtained mutants could not be isolated simply because they died before having progeny, maybe due to sterility phenomena because of the high mutagen dosis. Likewise, Paton and Sullivan (6) found that 5 of the 27 mutants isolated were viable as homozygotes, the remaining 22 were lethal when homozygous and were assumed to be associated with induced lethals on the second chromosome. The higher isolation frequency was obtained for sf gene. Aparisi & Nájera (16) also found a great number of sf mutants from natural populations in respect to the frequency of the other 4 genes studied.

The high mosaicism found in this work confirms that mosaicism is a characteristic of mutagenesis with alkylant compounds. Frequencies of mosaicism for bw, dke and Hn^r genes (between 30 and 42%) are similar to those obtained for other eye colour loci as w (17), cn (6) or lower than for other loci as dp (77%) (5) since mosaicism seems more evident when mutations are induced with EMS in loci which involve a high number of cells from the ancestor to constitute the phenotypically studied organ. So, this is the most logical reason for the higher frequency found in dp locus.

EMS acted primarily on postmeiotic sperm cells, the premeiotic and meiotic cells remaining relatively immune to its effects. In this work it was also shown that EMS had virtually no effect on premeiotic cells since only the 14.81% of the mutants appeared be-

tween the 5th-7th day after exposition and no mutant was obtained after the 7th day.

Complementation was not found among the mutants from the same locus. This suggests the existence of only one complementation class for each one of the five studied mutations. Likewise, Paton & Sullivan (6) do not found complementation in the production of eye colour among the cn examined mutants. Opposite, Mackay *et al.* (13) found that some Pu mutants were capable to complement almost completely for viability and for defects in the eye pigments appearing some heteroallelic combinations almost wild type. Also Reynolds & O'Donnell (7) found that some heteroallelic genotypes which allowed a low viability complemented almost completely for eye colour while others gave almost normal viabilities but low capacity to synthesize eye pigment.

The viability of the marker strain al b cn sp is very low and increases considerably when the EMS-induced mutation is introduced in the strain. This could be due to the removal of consanguinity in the strain when crosses were carried out in the mutant isolation scheme. In this work, the viabilities of the induced mutants at 25°C were higher than at 16°C but the phenotype was not changed. Heterozygotes showed higher viabilities than induced mutants. Other works about eye colour viabilities comparing homo- and heterozygotes in different situations (18), also showed this higher viability for heterozygous genotypes. In relation to the MDT a high variability among the alleles of the same locus and a trend to increase the MDT for the induced mutants can be observed.

The biochemical approximation to the chromatographic pattern and to the quantitative estimation of the red pigment of the induced mutants confirms the variability found in other works (16). An interesting mutant from the biochemical point of view is bw2MN with a chromatographic pattern similar to the bwV32g allele (19) but it is not lethal as in homozygosis. Its percentage of drospterins is about 20% of the wild Or-R level while the other natural mutant and the standard have a level of about 1.2-1.4%. dke3MN mutant has a significantly reduced amount of red pigment with respect to the standard mutant with decreased levels of sepiapterin and isoxanthopterin compared with the mutants from the natural populations measured by Aparisi & Nájera (16). Given that the EMS action over the genome occurs randomly, the frequency of mutation will be given so much for the size of the gene as for its importance for the viability of individual since the frequency of lethality or sterility will be higher if a mutation occurs in an important gene.

Results obtained in this work such as the low mutagenesis frequencies for Hn^r and se mutants and the fact that some metabolites are found totally absent (which seems indicative of the absolute blockade of a metabolic step) agree with the hypothesis of Bel and Ferré (1) according to which Hn^r and se can be structural mutants of the pteridine pathway. This pathway in *Drosophila* is very complex, with steps which are not really relevant (*bw*, *dke* and *sf* show a great variability that do not affect life parameters) and other steps that could be thought to be structural in the pathway (*sc* and Hn^r). Nevertheless, the performance of methods to measure the enzymatic activity will be definitive to help us to discern the existence of an estructural role of both genes.

In conclusion, to carry out mutagenesis experiments can be a very useful way to produce variability in loci we are interested and therefore a very important methodology in genetic and biochemical studies.

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