LINKAGE DISEQUILIBRIA IN TWO NATURAL POPULATIONS OF *D. subobscura*

by

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DECLARATION

I did this work and composed this thesis entitled: "Linkage Disequilbria in two natural populations of *D. subobscura*" by myself with the advice of my supervisor, Professor Alan Robertson.

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SUMMARY

Summary of experiments with D. subobscura, using isogenic lines extracted from two genetically different natural Greek populations and analysing the O chromosome and the enzyme loci Ao , Me , , Xdh and Odh located on it.

(A) Linkage disequilibrium observed between Xdh and Ao loci at the two tested populations

The linkage disequilibrium observed between the Xdh and Ao loci in the 1972 sample of both populations were the result of sampling or genetic drift and not the result of epistatic natural selection:

(1) After ten generations this linkage disequilibrium was not observed, nor was there any increase in frequency of the alleles of the favoured combination.

(2) The same is true after ten additional generations at the Parnes locality.

(3) In the Crete sample local genetic microdifferentiation was observed; therefore sampling can be biased.

(4) Null alleles and chromosomes carrying two copies were observed for the first time at both loci. This may cause inaccuracy in the estimation of gene frequencies.

(B) Hidden heat-sensitive polymorphism

We may conclude from these experiments that the heat-sensitive technique as it has been applied (crude extracts) is not as promising a method as it first appeared to be, for applications to population genetic studies:

(1) The heat-sensitive-electrophoretic phenotypes observed were not solely the result of products of the examined enzymatic loci.

(a) Heat-sensitive phenotypes were not repeatable in some tested strains (Table XIII).

(b) The heat-sensitive phenotypes within F_1 crosses were not homogeneous in most of the tested cases.

(c) In test crosses, using the null strains and single fly analysis, we did not observe one-locus Mendelian segregation.

(2) In the case of the Ao locus, the heat-sensitive polymorphism seems to be under the control of another genetic factor unlinked to the Ao structural locus (alleles Ao^2 and Ao^4).

(3) In the Me locus case, it seems that the heat-sensitive polymorphism is a result of peptidase action on the products of the Me locus. This could be very important because the peptidase can use as substrate any structural or enzymatic protein existing in the crude extract. We have to keep in mind that the peptidases in D. subobscura showed a lot of polymorphism (qualitative differences) and null allele (quantitative differences).

(4) If such a peptidase action is the rule (for these temperatures) we then increase (by a factor) artificially the hidden heat-sensitive polymorphism in a number of tested loci, even those which are monomorphic.

CONTENTS

INTRODUCTION

It is now established that a great amount of isozyme polymorphism exists in wild populations of organisms from viruses to man. Furthermore, as new methods from molecular genetics are applied to population genetic studies, undetected polymorphism will be revealed. The polymorphic variants are produced by mutation, but there is at present a controversy over the "forces" through which these variants are maintained in wild populations.

There are two diametrically opposing hypotheses which have been proposed to explain the above phenomena. One is the selection hypothesis, which says that some form of balancing selection is responsible for the maintenance of these polymorphisms. The other is the neutrality hypothesis, stating that the polymorphisms are due mainly to selectively neutral genes maintained by stochastic processes such as random drift.

Balancing selection may be in the form of overdominance, truncation selection or any kind of epistatic selection among loci. If any of these kinds of selection operate to maintain polymorphism a high degree of linkage-disequilibriumin equilibrium populations should be observed. According to the simulation results of Will et al. (1970) and Franklin and Lewontin (1970) only a few types of chromosomes should be maintained in an equilibrium population, so the level of linkage-disequilibrium becomes very high (although the genetic load problem is solved).

Apparent linkage-disequilibrium observed in samples from natural populations may have been generated by several mechanisms: simple random sampling of the populations, type I errors (mainly from the statistical use of the X^2 test for disequilibrium), epistatic natural selection and

genetic drift. Of these genetic drift is the only biological factor which produces linkage-disequilibriuml.d.) between genetic markers (enzymatic loci, inversions) in the populations.

This sort ofdisequilibriumcan be maintained in the populations for many generations before an equilibrium point will be approached, as first described by Hill and Robertson (1968) in their pioneering theoretical work. They estimated the $E(D^2)$, (expected variance of l.d.) when: (a) C (recombination function) is equal to zero (both markers in the same small inversion, for example), (b) large effective population size and (c) initial equilibrium, through the formula:

$$E(D^2) = \frac{1}{15} p_0(1-p_0)q_0(1-q_0)\{6(1-\bar{F}) - 5(1-\bar{F})^3-(1-\bar{F})^6\} \qquad (1)$$

where: p_0, $1-p_0$, q_0 and $1-q_0$ are the gene frequencies at equilibrium and $\bar{F}$ is the inbreeding coefficient of the population. Later this subject was described by Kimura and Ohta (1971, page 109) when the C is different from zero. They finally presented the formula:

$$\sigma^2 d \simeq \frac{1}{4R} \qquad (2)$$

where: R is equal to Ne.C (Ne = effective population size) and $\sigma^2 d$ is the normalized value of $E(D^2)$, used by Hill and Robertson. This $\sigma^2 d$ can be estimated from $E(D^2)$ or <u>vice versa</u> through the formula:

$$\sigma^2 d = \frac{E(D^2)}{E(p.q(1-p)(1-q))} \qquad (3)$$

The formula (2) can be used when (a) R = Ne.C >>1 and for all R > 1 and (b) the population is in a state of steady decay. The square root of $\sigma^2 d$ (σd) has been termed as the standard linkage deviation. This is very useful for quantitative linkage disequilibria estimations due to genetic drift.

Linkage-disequilibrium $(D^{t}_{AB})_{n}$ due to genetic drift will be decreased by a proportion approximately 1-C every generation, approaching asymptotically the equilibrium point D = 0, when no other restrictions in population size occur, according to the formula:

$$D_{AB(t)} = (1-C_{AB})^{t} \cdot D_{AB(O)} \qquad (4)$$

This formula gives the linkage disequilibrium $(D_{AB(t)})$ between the markers A and B after t generations regardless of the linkage disequilibrium at generation zero $(D_{AB(O)})$.

Investigations of natural selection using linkage-disequilibrium can be classified into two categories. The first is the spatial method; according to this method, samples are taken from many genetically different populations and tested for the available genetic markers to investigate the existent linkage-disequilibria. The second is the temporal method; linkage-disequilibria estimated already are observed for future changes. If, after successive generations, these linkage-disequilibria still exist or the discrepancy from the equilibrium point is greater, this is a kind of evidence that these l.d. were the result of epistatic natural selection (see theoretical work by Avery and Hill (1979). The authors by using symmetric fitness models estimate the distribution and stability of l.d. in finite populations with selection). If, however, these l.d. disappear this will be evidence for genetic drift. The conclusions of the temporal method are valid given the following conditions: (a) that no new restrictions in effective population size occur, (b) that the selection does not change in action, direction or intensity, and in addition that the recombination fraction (C) must be quite high.

At this point I will briefly mention the two more extensive l.d.

studies among enzymatic loci in D. melanogaster populations, noteworthy both because of the large number of observations and of the application of both the spatial and temporal methods.

The first is described by Mukai (1978) and was carried out by Mukai et al. (1971), Mukai et al. (1974), Mukai and Voelker (1977). They investigate and follow linkage-disequilibria from 1968 to 1974 at the Raleigh, North Carolina population. Second chromosomes were extracted using the balanced strain $\frac{In(2LR)\ SM_1(Cy)}{In(2LR)\ bw\ V_1(Pm)}$ in 1968, 1969, 1970 and 1974 samples.

The sample sizes were 314, 146, 698 and 617 respectively. The effective population sizes were 24,000, 22,000, 115,000 and 18,000, estimated by using Nei's (1968) formula. These number are surprisingly large. The markers used were aGpdh (2,17.8), Mdh (2,35.3), Adh (2,50.1), Hex-C (2,74.5), Amy (2,77.3) and two inversions In(2L)t and In(2R)NS.

I copy here a part of Mukai's (1977) table:

Combination	1968	1969	1970	1974
aGpdh-vs-Mdh	+0.083	-0.043	-0.168***	-0.052
Mdh-vs-Amy	-0.155**	-0.083	+0.008	-

The table represents the correlation of gene frequencies and their significance: (**) significant at the 1% level, (***) highly significant.

From these results the major conclusions from this study are:

(a) The l.d. detected between isozyme genes are sporadic.

(b) These l.d. do not continue over successive years.

(c) The apparently significant l.d. detected by using the X^2 test in the 1974 sample is an artifact of using this method (X^2 test).

The second experiment was done by Langley et al. (1978). They used as markers eight polymorphic enzymatic loci in the same species (D. melanogaster) (aGpdh (2,20.5), Mdh (2,37.2), Adh (2,50.1), Est6 (3,36), Pgm (3,43.3), Odh (3,49.2), Est-C (3,51.7), Acph (3,101.1)) and examined more than a hundred samples from natural and two cage populations in space and time.

In this experiment the zygotic frequencies were analyzed. The analysis of linkage-disequilibrium from zygotic frequencies can be done in several ways: e.g. Hill (1974) suggested an approach through which all deviations from random assortment to zero are set and then maximum likelihood techniques are applied. The same author developed algorithm for obtaining the maximum likelihood estimate for comparisons. Another method suggested by Bodmer et al. (1969) is by estimating the overall covariance of non-allelic genes in individuals, but this method makes no assumption about inbreeding or higher order deviations from random assortment.

The authors use another estimate from their formula

$$\Delta = \tfrac{1}{2}\{4f(^{11}_{11})+2f(^{11}_{01})+2f(^{11}_{10})+f(^{11}_{00})\}-2p_1p_2 = D+T$$

where D is the coefficient of linkage disequilibrium (covariance between non-allelic genes within gametes) and T is the covariance between non-allelic genes in uniting gametes. p_1 and p_2 are the allele frequencies of the "1" allele at the two loci respectively, $f(^{11}_{11})$ is the frequency of double homozygotes for the "1" alleles, $f(^{11}_{01})$ is the frequency of zygotes heterozygous at the first locus and homozygous at the second locus for the "1" allele.

The conclusions from this analysis are summarized below:

(a) The magnitude of linkage disequilibria was small.

(b) The magnitude of linkagedisequilibriumwas observed to be positively correlated with linkage.

(c) The two cage populations were observed to demonstrate larger amounts of linkagedisequilibriumbetween closely linked loci than in natural populations.

(d) Occasionally individual instances of disequilibrium were observed. These were sporadic and never consistent in time or space. The similarity between the results of the two experiments is obvious and even more striking given that the first experiment utilizes gametic and the second zygotic analysis.

In these two large scale experiments and in every other to this date (to my knowledge at the time of writing) the amount of l.d. observed among enzymatic loci is too small to be consistent with the predictions of Wills et al. (1970) and Franklin and Lewontin (1970).

I want to stress once more that the standard deviation of the expected linkage-disequilibria due to drift, as estimated theoretically by Hill and Robertson (1968) (for $C = 0$) and Kimura and Ohta (1971) (for $C \neq 0$), is very large in comparison to the actual amount of l.d. observed experimentally; thus the methods employed are too insensitive to detect any natural selection which may be acting.

Only the temporal method is potentially useful in the detection of the action of genetic drift by following already observed l.d. through successive time periods.

In 1973 I decided to apply the temporal method to the already observed l.d. case in D. subobscura (Zouros and Krimbas, 1973).

These authors observed l.d. between the loci Xdh (Xanthine dehydrogenase) and Ao. (Aldehyde oxidase), in samples from two

genetically different natural Greek populations, Parnes (P) and Crete (K), in D. subobscura.

To determine whether these l.d. were due to genetic drift or were a result of epistatic natural selection, samples were taken from the same populations and localities after approximately ten generations and isogenic lines extracted for the O chromosome on which these loci are located.

Isogenic lines were constructed because from these we obtain better estimates of gene frequencies if null alleles exist or chromosomes with two alleles (duplicated copies), and because the chromosomal analysis is simplified by reading only one slide (salivary chromosomes) from the F_1 crosses, which are the result of crossing the isogenic strains with another strain having known chromosomal structure. In addition we wanted to estimate lethality and allelism of these two populations for other purposes.

In order to increase the sensitivity of my estimation, I decided not only to use the hidden-heat-sensitive polymorphism (see below) but also two more polymorphic loci located close to the tested pair. These are Me. (Malic-enzyme) and Odh (n-Octanol-dehydrogenase).

The map position of the four tested loci is as follows: (in order from the centromere to the tip chromosomal end see Figure 2 for more details).

Odh (50.6), Ao. (55.9), Me. (83.5), Xdh (87.3)

The map distance between the loci Ao. and Xdh is 21.4 so the recombination fraction C (genetic sex ratio is 1 to 1) in the populations, corrected by the I.F.R. coefficient (Inversions-Free- Segment part, see Table V1), are 10.4 for Crete and 10.2 for Parnes.

These values are sufficient to eliminate the observed l.d. after ten generations according to formula (4).

Hidden heat-sensitive polymorphism

It is known that the amount of polymorphism which is detectable by ordinary electrophoresis may be only a proportion of the total. Of all aminoacid substitutions, we can investigate through ordinary electrophoresis only those which give a different electric charge and mobility of the alloenzyme molecule. This proportion is approximately thirty percent of the total.

The task of recent experimental work in this field was to determine how to reveal this hidden polymorphism by using simple variations applied to the electrophoretic techniques in population genetic studies and to use these as more sensitive tools to investigate natural selection and its operations.

The main agents used for this purpose were: temperature, separation by the isoelectric point, different pH values, varying concentrations of acrylamide or starch in the gels or by the addition of chemical compounds (e.g. S.D.S. urea, pCMSA (p-chloromercuriphenylsulphonic acid) which give the enzyme different electric charge, stability or activity according to its aminoacid composition.

A number of publications have appeared in the last years in which such hidden polymorphism was revealed by using heat-treatment of several enzymatic loci in different Drosophila species.

The reason for using heat denaturation was not only the simplicity of the technique but also because temperature is one of the main environmental parameters determining the ecology of Drosophila (see, for example: Singh, Lewontin and Felton (1976); Bernstein, Throckmorton and Hubby (1973); Pandey (1972); Singh, Hubby and Throckmorton (1975); Trippa, Leverse and Catamo (1976); Cochrane (1976); Singh, Hubby and Lewontin (1974)).

In most of these studies the genetic material used was isofemale lines or inbred strains with a high degree of homozygosity. The technique used in this work was as follows:

From every strain, eight to twelve flies were homogenized in a small tube, the extract was centrifuged, the supernatant equally separated into four small tubes (samples). One of these tubes did not receive heat-treatment and was used as a standard, while the other three were placed in an oil or water bath with empirically selected temperature for different periods of time. All four samples from each strain were then analysed by ordinary electrophoresis (see, for example, Table IX).

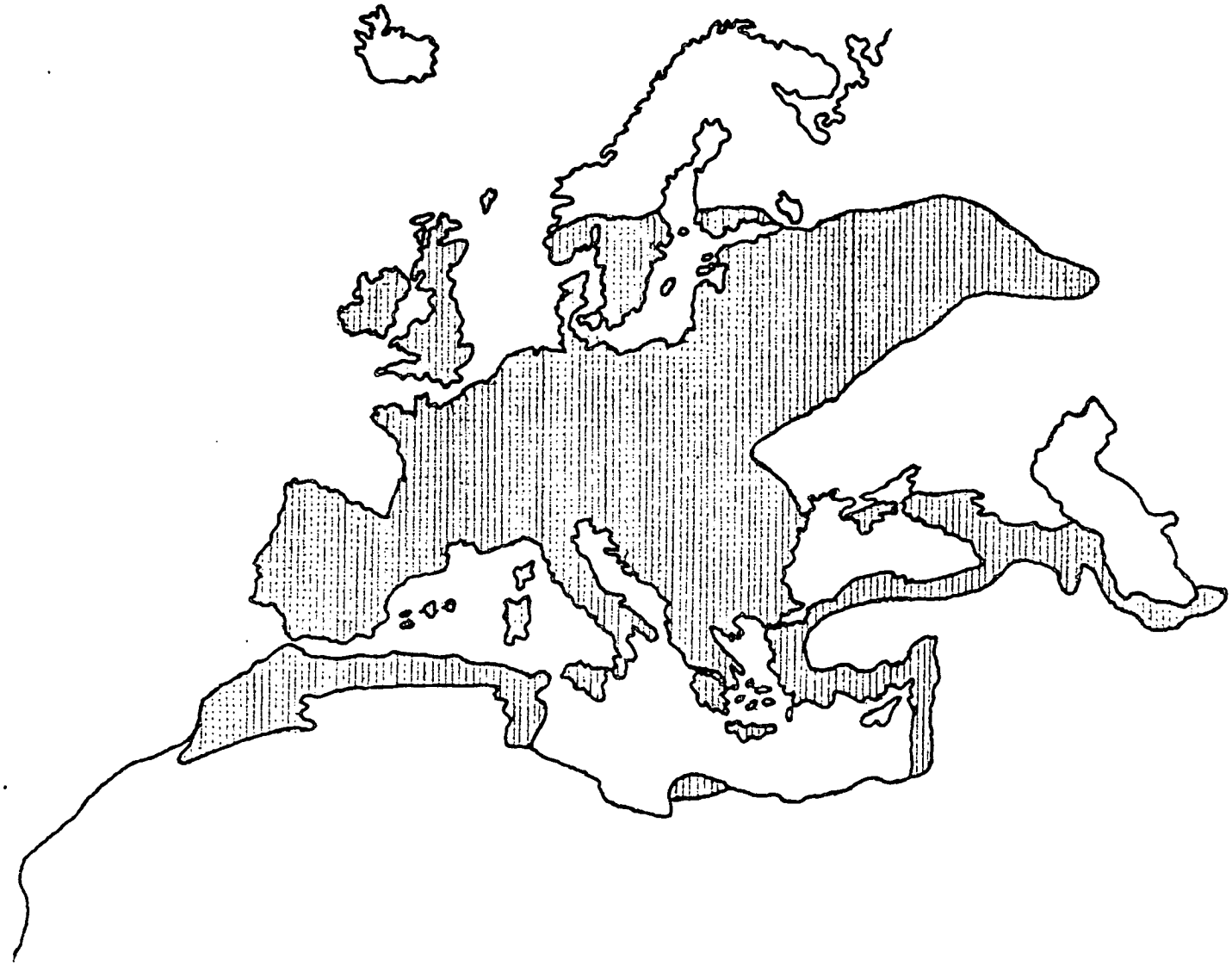
Using this technique it was possible to detect from two different strains, alleles having the same electrophoretic mobility but different heat resistance(/the first one losing its activity on the gel after five minutes of heat-treatment while the second from the other strain remains active). e.g.

A possible hypothesis is that among the two enzyme molecules there are such aminoacid differences as to give both the same electrophoretic mobility but different heat resistance.

The evidence supporting the hypothesis that hidden polymorphism was revealed by this technique includes:
(a) the repeatability of the heat-sensitive phenotypes of the strains and (b) that samples from the F_1 crosses between strains reveal the same electrophoretic and heat-sensitive pattern. See for example, Trippa et al. (1976) for experiments with the alleles Pgm^A, Pgm^B, Pgm^C and Pgm^D of the Pgm locus using F_1 and F_2 in D. melanogaster; Cochrane (1976) on the other hand, in the same species and for the Est-6 locus came to the conclusion by using F_1 and F_2 crosses that

it is questionable whether the observed heat-sensitive polymorphism is a property of the Est-6 locus product and not the result of another locus (modifier) interaction linked to the Est-6 structural one.

The purpose of my experimental work was to follow temporally the previously observed l.d. in D. subobscura utilizing electrophoretic and heat-sensitive polymorphism of the loci Odh, Ao , Xdh, Me .



MAP 1. Geographical distribution of Drosophila subobscura (Loukas et al. 1978)

MAIN EXPERIMENTS

(a) Homologies

The species D. subobscura belongs to the obscura group and to the obscura subgroup as first described by Collin (1936). The approximate geographical distribution is given by Map 1. The mitotic chromosomes of this species consist of five pairs of acrocentric chromosomes plus a small pair (Dot). The chromosomal homology of this species with D. melanogaster and other species from the obscura group (D. pseudoobscura, D. affinis) is as denoted in Table I, Patterson and Stone (1952), Buzzati-Traverso and Scossiroli (1955), Sondhi (1957).

TABLE I

Chromos.	D. subobscura	D. melanogaster	D. pseudoobscura D. affinis
X	A	X	XL
2	J	3L	XR
3	U	2L	4
4	E	2R	3
5	O	3R	2
6	Dot	IV	5

The chromosomal homology among the species D. subobscura, D. melanogaster and D. pseudoobscura according to the electrophoreticenzymatic loci and their chromosomal positions is shown in Table II. The homology between the chromosomal arm 3R of D. melanogaster and the O chromosome of D. subobscura is presented in Table III (O'Brien and McIntyre (1971), Koske and Maynard Smith (1954) and experimental unpublished work from Department of Genetics, Agricultural College of Athens).

TABLE II

Homology between chromosomes according to electrophoretic-enzymatic-loci and for the species D. subobscura, D. melanogaster and D. pseudoobscura (from Loukas et al., 1978)

	D. subobscura	D. melanogaster	D. pseudoobscura
HK	E	2R	..
Amy	..	2R	3
Est-9	E	2[1]	..
Adh	U	2L	4
Mdh	U	2L	4
aGPDH	U	2L	..
Aph	J	3L	X[2]
Est-7	J	3L(Est-6?)	X[2] (Est-5)
Idh	J	3L	..
Pgm	J	3L	..
Est-5	O	3R(aliesterase?)	..
Est-8	O	3R(Est-C?)	..
Odh	O	3R	2
AO	O	3R	2
Xdh	O	3R	2
ME	O	3R	2
Lap	O	3R(LapA?,LapB?)	2
Acph	O	3R	..
6-PGD	A	X	XL
G-6-PGD	A	X	..
shv(short vein)[3]	Dot	4(ci=cubitus interruptus)	..

TABLE III

D. melanogaster 3R arm		D. subobscura O chromosome	
Map position	Locus	Map position	Locus
48	aliesterase	13.5	Est-5
49	Est-C	19.2	Est-8
49.2	Odh	50.6	Odh
50	Cu (Curled)	52.6	Cu (Curled)
52	Xdh	87.3	Xdh
53.1	Me	83.5	Me
56.6	Ao	55.9	Ao
58.5	SS^{α} (spineless aristapedia)	35.5	(antemapedia aristapedia)
66.2	Delta		Va (variable)
69.5	H (Hairless)	54.7	Bare
75	Ed (Cardinal)	98.2	Ch (Cherry)
98	Lap-A	213.2	Lap
98.3	Lap-D		
101.1	Acph-1	228.3	Acph

The loci sequence in D. subobscura on the O chromosome from the centromere to the tip end is as follows:

Est_5-Est_8-Va-ant,ar-Odh-cu-Ba-Ao-Me-Xdh-Ch-Lap-Acph.

This sequence differs from the D. melanogaster 3R arm sequence by three inversions which occurred successively from 3R to O as follows:

Chromosomes

3R — al-EstC-Odh-cu-Xdh-Me-Ao-SS^{α}-Dl-H-cd-Lap-Acph

1st inversion

"$3R_{,}$ — al-EstC-Dl-SS^{α}-Ao-Me-Xdh-cu-Odh-H-cd-Lap-Acph

2nd inversion

"$3R_{,}$ — al-EstC-Dl-SS^{α}-Odh-cu-Xdh-Me-Ao-H-cd-Lap-Acph

3rd inversion

"$3R_{,}$ — al-EstC-Dl-SS^{α}-Odh-cu-H-Ao-Me-Xdh-cd-Lap-Acph

O — Est5-Est8-Va-ant,ae-Odh-cu-Ba-Ao-Me-Xdh-ch-Lap-Acph

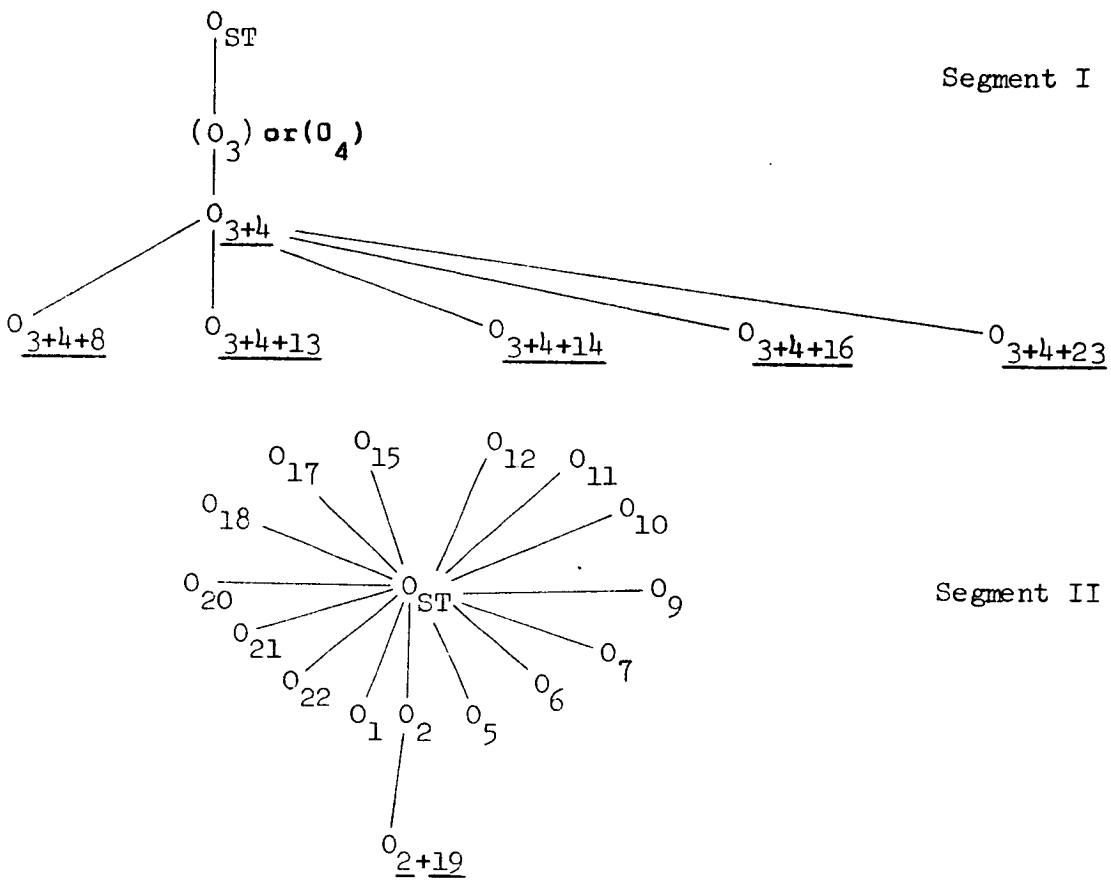


Figure 1. Phylogenetic relation between different inversions of the segments I and II of the O chromosome. **(Loukas and Krimbas, 1979).**

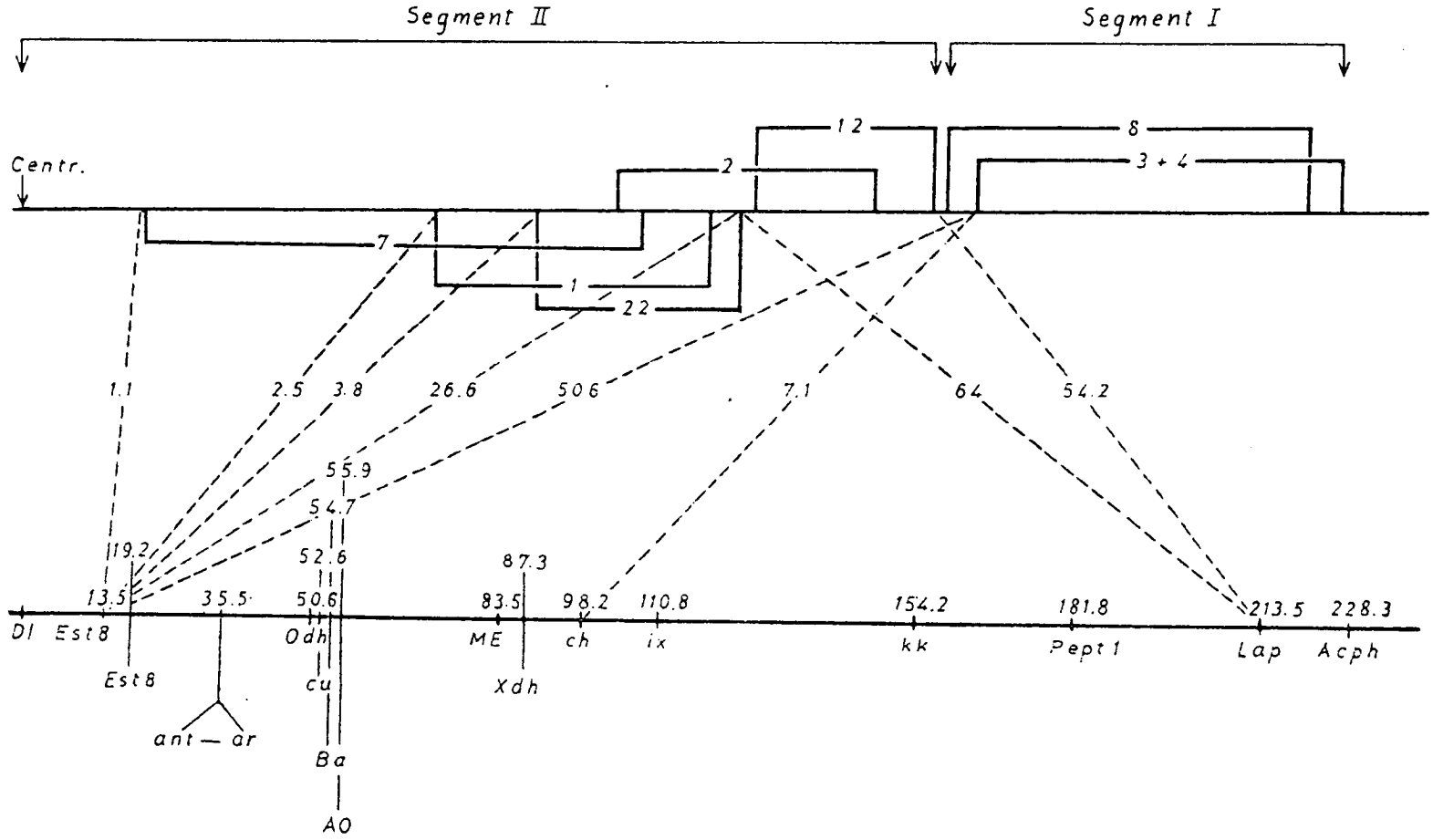


Figure 2. Cytological and genetical map of O chromosome. Xdh and Ao . are located in the inversions O_1, O_{22} (Zouros and Krimbas (1973), Krimbas and Loukas (1979)).

TABLE IV

Inversions and Duplications of D. subobscura salivary chromosomes

Name	Breaking points	Author*	Chromosomal structure
A_1 (=Attila)	2A/2B - 7C/7D	KM-M	A_{ST}
A_2 (=Aldo)	8C/8D - 12A/12B	KM-M	A_{ST}
	8C/8D - 12C/12D	G	
A_3	8D/8E - 16C/16D	G	A_2
A_4	3C1/3C2(3C/3D?)-11D/12A	J-1	A_2
A_5	1C1 - 4C1	J-1	A_{2+3}**
A_6	1C/2A(2A/2B?)-7D/8A(7C/7D?)	J-1	A_2
A_7	1C1/4C1 - 7D3/8A(7C/7D?)	J-1	A_{2+3+5}
J_1 (=Ingrid)	22B/22C - 25A1/25A2	KM-M	J_{ST}
J_3	21D/22A - 25A1/25A2	KM-M	J_{ST}
J_4	24D/25A - 29B/29C	KM-M	J_3
U_1 (=Urs)	39D/40A - 45E/46A	KM-M	U_{ST}
U_2 (=Ursula)	45E/46A - 51C/51D	KM-M	U_{ST}
U_3 (=Undine)	50B/50C - 52A/52B	KM-M	U_{1+2}
U_4	45A/45B - 50A/50B	KM-M	U_{1+2}
U_5	40D/41A - 48C/48D	KM-M	U_1
U_6	45A/45B - 49C/49D	KM-M	U_{1+2}
U_7	40A/51C - 52A2/52A3	KM-M	U_{1+2}
U_8 (=Ursula)	40A/51C - 44D/45A	KM-M	U_{1+2}
U_9		Gdl	U_{1+2+7}
U_{10}	49D/50A - 52C	G	U_{ST}
U_{11}	45A/45B - 48C/48D	G	U_{ST}
$U_{DUPLICATION}$		G	U_{ST}
E_1 (=Edda)	58D/59A - 62D/63A	KM-N	E_{ST}
E_2 (=Eleonore)	58D/62D - 64B/64C	KM-M	E_1
E_3	58D/68B - 64C/62D	KM-M	E_{1+2+9}
E_4	65C/66A - 64B/68C	G	E_{1+2+9}
E_5	61C/61D - 64B/69C	G	E_{1+2+9}
E_8 (=Emanuel)	61C/61D - 68D/68E	KM-M	E_{ST}
E_9 (=Ernst)	58D/68B - 64B/64C	KM-M	E_{1+2}
E_{12} (=Eva?)	67B/67A - 61D/61C	KM-M	E_{1+2+9}
E_{13}	69C/70A - 71C/72A	KM-M	E_{1+2+9}
E_{14}	68A? - 70D?	K	E_{ST}
E_{15}	54C - 67A/67B	J-2	$E_{1+2+9+4}$

TABLE IV (continued)

Name	Breaking points		Author*	Chromosomal structure
O_1 (=Oswald)	82B/82C	- 86D/86E	KM-M	II_{ST}
O_2 (=Ottokar)	85C/85D	- 89B/89C	KM-M	II_{ST}
O_3 (=Orson)	91B/91C	- 94E/95A	KM-M	I_{ST}
O_4 (=Ottilie)	94E/98C	- 98C/98D	KM-M	I_3
O_5 (= Othello)	83B/83C	- 87D/87E	KM-M	II_{ST}
O_6 (=Ossian)	85B/85C	- 90C/90D	KM-M	II_{ST}
O_7 (=Oskar)	77B/77C	- 85E/86A	KM-M	II_{ST}
	77B/77C	- 85E	G	
O_8 (=Olga)	90D/91A	- 94A/94B	KM-M	I_{3+4}
O_9	83C/94A	- 91B/91C	KM-M	II_{ST}
O_{10}	83B/83C	- 86D/86E	G	II_{ST}
O_{11}	86A	- 87E/88A	G	II_{ST}
O_{12} (=Ortega)	87C/87D	- 90C/90D	KM-M	II_{ST}
O_{13}	93A/93B	- 99C	KM-M	I_{3+4}
O_{14}	91A/91B	- 92C/92D	G	I_{3+4}
O_{15} (=Othmar)	83C/84A	- 91A/91B	KM-M	II_{ST}
O_{16}	96A/96B	- 98D/99A	KM-M	I_{3+4}
O_{17}	81B/81C	- 87A/87B	KM-M	II_{ST}
O_{18}	85C/85D	- 90A/90B	KM-M	II_{ST}
O_{19}	93B/93C	- 99A/99B	KM-M	II_2
O_{20}	79B/79C	- 85C/85D	KM-M	II_{ST}
O_{21} (=Orest)	83C/84A	- 91B	Gd-2	II_{ST}
O_{22}	83C/84A	- 87A/87B	KM-M	II_{ST}
O_{23}	96A/96B	- 99A/99B	J-1	I_{3+4}
$O_{DUPLICATION}$			G	I_{ST}

(*) KM-M : Kunze-Mühl, E. and E. Müller, 1958 Weitere Untersuchungen über die chromosomale Struktur and natürlichen Strukruktypen von Drosophila subobscura. Chromosoma 9, 559-570.

G : Götz, W., 1965 Beitrag zur Kenntnis der Inversionen, Duplikationen und strukturtypen von Drosophila subobscura. Coll. Z. Vererbungsl. 96, 285-296.

Gd-1 : Goldschmidt, E., 1958 Polymorphism and coadaptation in natural populations of Drosophila subobscura. Proc. Xth Inter. Congr. Ent. 2, 821-828.

TABLE IV (continued)

(*)

Gd-2 : Goldschmidt, E., 1956 Chromosomal polymorphism in a population of Drosophila subobscura from Israel. J. Genetics 54, 474-496 (p.477).

J-1 : Jungen, H., 1968 Inversions polymorphisms in tunesischen Populationen von Drosophila subobscura Collin. Archiv. Julius Klaus-Stiftung 43, 1-55.

J-2 : Jungen, H., 1967 Evidence of spontaneous inversion in D. subobscura DIS 42, 108.

K : Knight, G.R., 1961 Structural polymorphism in Drosophila subobscura. Coll. from various localities in Scotland. Genet. Res. 2, 1-9.

(**) The underlined notation at some chromosomal structures shows overlapping inversion.

(***) This table is taken from the work of Loukas and Krimbas (1979)

TABLE V

Inversion frequencies of the two populations, Parnes and Crete (Zouros et al., 1974; Krimbas, 1965 and Krimbas, 1967).

	Parnes	Crete
A_{ST}	0,466	0,435
A_1	0,256	0,130
A_2	0,278	0,435
N	708	162
J_{ST}	0,158	0,093
J_1	0,837	0,432
J_{3+4}	0,005	0,475
N	1517	162
U_{ST}	0,017	0,006
U_1	0,003	0
U_2	0,009	0,241
U_{1+2}	0,346	0,074
U_{1+2+4}	0	0
U_{1+2+6}	0,472	0,074
U_{1+2+7}	0,112	0,006
U_{1+2+8}	0,033	0,599
U_{1+2+3}	0,007	0
N	1516	162
O_{ST}	0,126	0,038
O_5	0,002	0
O_6	0,001	0
O_7	0,002	0
O_9	0,001	0
O_1	0,001	0
O_{3+4}	0,463	0,076
O_{3+4+1}	0,229	0,045
O_{3+4+2}	0,037	0,006
O_{3+4+6}	0	0
O_{3+4+7}	0,048	0
O_{3+4+8}	0,014	0,822
O_{3+4+9}	0,001	0
O_{3+4+17}	0,003	0
O_{3+4+22}	0,072	0,013
$O_{3+4+16+2}$	0,001	0
N	1531	157

CONTINUED/

TABLE V (Continued)

	Parnes	Crete
E_{ST}	0,115	0,054
E_{8}	0,250	0,059
E_{1+2}	0,061	0,667
E_{1+2+9}	0,548	0,186
$E_{1+2+9+12}$	0,025	0,029
$E_{1+2+9+3}$	0,001	0,005
N	1687	204

(b) Inversions

Up to now 58 inversions and two small duplications in D. subobscura have been described, and are presented in Table IV. The kind of inversions and their frequencies between the two sampled populations, Parnes and Crete, is given as Table V (Zouros and Als (1974), Krimbas (1965), Krimbas (1967)). The phylogenetic relation between inversions of the O chromosome at the segment I (tip end) and at the segment II (top end or centromere side) is indicated by Figure 1.

Table VI presents the I.F.R. (Inversions Free Segment) values of the two populations for all chromosomes and for the O chromosome according to Carson (1955).

TABLE VI. Values of I.F.R. for the two Populations

Popul.	Chromosomes					I.F.R.	
	E	U	J	O	A	All chromos.	O chromos.
Parnes	4.89	5.41	0.90	4.60	3.83	80.37	95.4
Crete	3.94	3.78	4.90	2.48	3.44	82.16	97.52

These values for the O chromosome in which we are interested are very high in both populations. The cytological and genetical maps of the O chromosome are presented in Figure 2.

(c) Balanced strain for the O chromosome

The balanced strain for the O chromosome, a tool to produce isogenic lines, was given to us by Professor D. Sperlich (Tübingen).

One chromosome of the pair contains the inversions O_{3+4}, x+y and the visible mutations Va (Varicose, dominant lethal), ch (cherry, recessive) and cu(curled, recessive). The other has standard

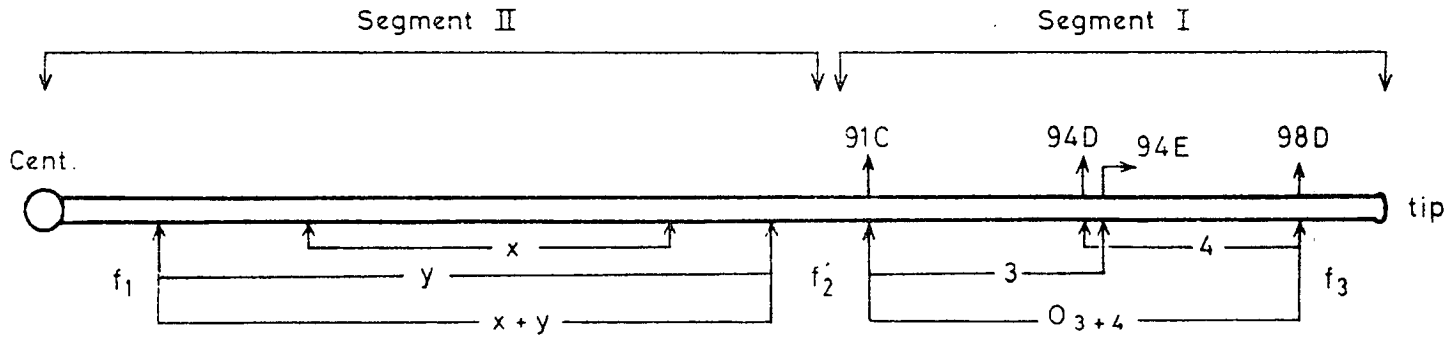


Figure 3. Chromosomal structure of the inverted O chromosome of the balanced strain ($\frac{\text{Va ch cu}}{\text{Ba}}$): f_1, f_2, f_3: unknown in length free recombination segments when the other chromosome has OST structure. The loci Odh, Xdh, Me . and Ao . probably located in the overlapping inversions x+y; the loci Lap, pept 1 in the O_{3+4} inversions. (Loukas et al. 1978)

chromosomal structure (OST) and carried the dominant lethal Ba (Bare).

The inversions x+y were produced after U.V. irradiation. From cytological and genetical observations it is known that: (a) The inversions O_{3+4}, x+y cover a large region of the chromosome, (b) If the other (wild) chromosome carries the inversion O_{3+4} and not the O_{3+4+8} the segment I of this chromosome is not protected from crossing-over, on the contrary, segment II is well protected in all cases (Panopoulou, 1973).

The area where the loci Odh, Ao., Me. and Xdh are located are protected from crossing-over (C-O) by the overlapping big inversions x+y. Only cytological observations have been made thus far. These are presented in Figure 3.

The genetic lengths of the free inversions chromosomal segments f_1, f_2, f_3 are not known even when the other chromosome of the pair has an OST structure.

(d) Techniques

(d.1) Isogenic lines

Every single wild type male trapped at Crete and Parnes locations was used for one isogenic line. The isogenic lines produced were 56 for Crete and 76 for Parnes. The extraction procedure was as described by R. C. Lewontin (1974, pages 38, 39).

(d.2) Electrophoretic analysis for the loci Odh, Ao, Me. and Xdh

For enzymatic detection I used the following buffer solutions as described by Burstone (1962, pages 574-584).

(A) .25M Tris-HCl pH = 8.6

(B) .1M Tris + .1M Maleic acid + 0.1M EDTA 2Na+ + 0.01 $MgCl_2$

pH = 7.4.

TABLE VII Buffer solutions, substrates, dyes, coenzymes and chemical reactions for electrophoretic detection of loci, Odh, Ao., Me and Xdh.

Enzyme	Gel Buffer	Tunks-Buffer	Staining buffer	Substrate	Dye	Coenzymes
Odh	C	D	E	n-octanol	N.B.T.(1) +P.M.S.(2)	NAD^+(3)
Ao.	C	D	E	benzaldehyde	N.B.T. + P.M.S.	-
Me	C	D	E	l-malic-acid	N.B.T. + P.M.S.	$NADP^+$(4)
Xdh	C	D	E or F	Hypoxanthine	N.B.T. + P.M.S.	NAD^+

CHEMICAL REACTIONS

Odh	$C_7H_{17}CH_2OH + NAD^+ \xrightarrow{Odh} C_7H_{17}CHO + NADH + H^+$ $\hookrightarrow$ PMS → NBT Phormazane → (blue precipitate)
Ao.	$C_6H_5CHO + H_2O \xrightarrow[-H_2]{Ao.} C_6H_5COOH$ PMS → NBT → Phormazane → (Blue precipitate)
Me.	l-malic-acid + $NADP^+$ $\xrightarrow{Me.}$ pyruvic acid + CO_2 + NADPH + H^+ → PMS → NBT → phormazane (>>)
Xdh	or Hypoxanthine + $NADP^+$ $\xrightarrow{Xdh}$ xanthine + NADH + H^+ Xanthine + $NADP^+$ $\xrightarrow{Xdh}$ ouric acid + NADH + H^+ $\xrightarrow{or}$ P.M.S. - NBT → phormazane (>>)

(1) Nitro-blue tetrazolium (30 mg/200 ml buffer)

(2) Phenazine-Metho-Sulphate (10 mg/200ml buffer)

(3) Nicotinamide-adenine-dinucleotide (20 mg/200ml buffer)

(4) Nicotinamide-adenine dinucleotide phosphate (20mg/200ml Buffer)

(C) .52M Tris + .01M Citric acid pH = 8.7

(D) .152M H_3BO_3 + .062M NaOH pH = 8.4

(E) .1M Tris-HCl pH = 8.5

(F) .1M Tris-HCl pH = 7.5.

The same buffer system was applied to the four tested loci throughout this experiment. Buffer C was used for the starch gel preparation and 4.5 g/l of EDTA. 2Na was added in the anlaysis of Xdh and Ao. Buffer D was used in the tanks. Electrophoretic conditions: U = 400 volts, i = 200 mA. The electrophoresis was completed when the front brown buffer line reached a distance of 12 cm. I followed the electrophoretic technique described by Zouros et al. (1974). The methods for enzyme detection are presented in Table VII.

The allelic frequencies of the loci Xdh and Ao reported by myself (unpublished), Zouros and Krimbas (1973) and Loukas et al. (1978) are presented in Table Xa. I chose to use a different buffer system because it gives faster analysis, better contrast in staining, and sharper bands. The alleles analysed in my work are certainly the same as were identified by the other workers because I analysed under the new conditions the same strains as were previously classified by Zouros and Krimbas and observed corresponding electrophoretic variants. The only difference is that I used a simpler nomenclature. I present at the end of Table IX the correspondence between the three nomenclatures.

(d.3) Heat-Treatments

(d.3.1) Mass Extract (12 adult flies)

Crude extract was taken from twelve (5 day old) adult flies in .4ml of distilled water in a small tube. Centrifugation followed at

3,000g for 3 minutes and the supernatant was separated equally into four small tubes.

The first tube was placed in the refrigerator as the control and the other three were put into an oil-bath (Shell No. 10) for heat-treatment of 5, 10 and 15 minutes duration respectively at empirically selected temperatures for each enzyme.

The temperatures were $72^{o}C \pm 0.5$ for Xdh and Ao, and $54^{o}C \pm 0.5$ for Me and Odh.

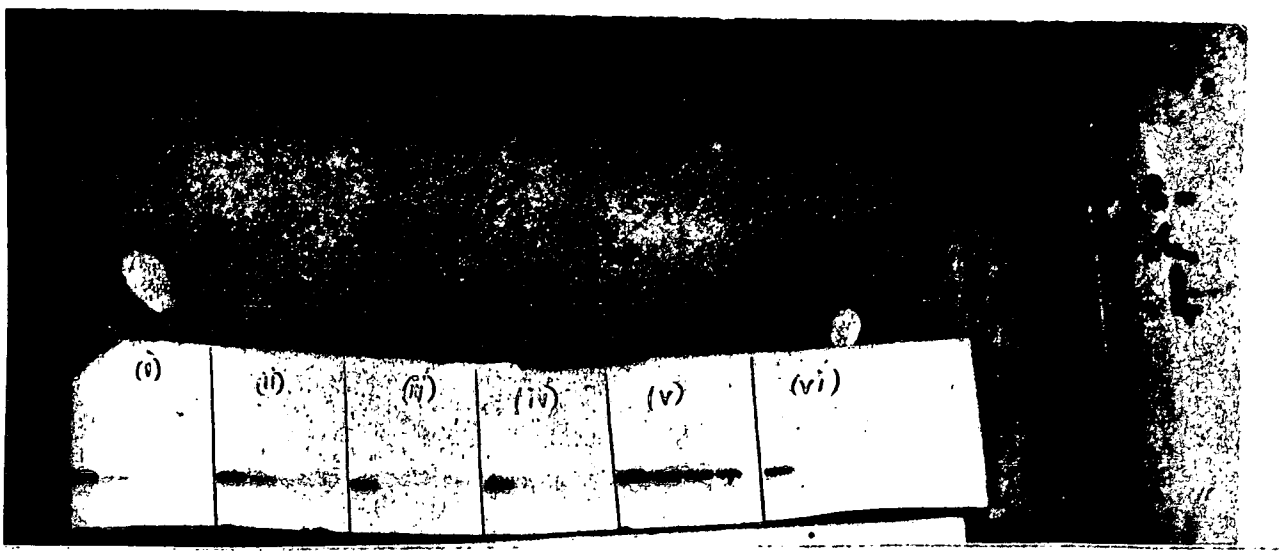
After heat-treatment each of the four samples was absorbed in a piece of filter paper (Whatman No. 3); these were placed in the starch gel near each other and in the order 0', 5', 10', 15'.

Electrophoresis followed; the gels were then stained and read.

(d.3.2) Single fly extract

One (5 day old) female fly, was homogenized in a small drop of distilled water (20λ) and four samples were taken by using cellulose-acetate papers (dimensions 0.5 x 0.3cm). These were then dropped into four tubes containing oil (Shell No. 10) which covered the slide

papers. One was placed in the refrigerator as the control and the other three in the oil-bath for heat-treatment followed by electrophoresis, staining and reading of the heat-sensitive-electrophoretic phenotypes (see Photograph 1).



Photograph 1. Six isogenic strains are tested. The reading from left to right is as follows: (i) Me^{1} contr.$^{+}$, 5'$^{-}$ 10'$^{-}$, 15'$^{-}$ (ii) Me^{1} contr.$^{+}$, 5'$^{+}$, 10'$^{-}$, 15'$^{-}$ (iii) Me^{2} contr.$^{+}$, 5'$^{-}$, 10'$^{-}$, 15'$^{-}$ (iv) Me^{2} contr.$^{+}$, 5'$^{-}$, 10'$^{-}$, 15'$^{-}$ (v) Me^{1} contr.$^{+}$ 5'$^{+}$ 10'$^{+}$ 15'$^{+}$ (vi) Me^{1} contr.$^{+}$, 5'$^{-}$ 10'$^{-}$ 15'$^{-}$

(e) Chromosomal analysis

Investigation of chromosomal structure took place after reading one salivary-chromosome slide from every F_1 cross between one male from every isogenic line with one virgin female from the strain (ch, cu) which has O_{3+4}/O_{3+4} chromosomal structure.

(f) Materials

Two samples of D. subobscura were collected (Spring, 1974): one from Parnes mountain (altitude 900m, 30kms north-west of Athens) and the other from Crete island near the town of Iraclion and the

village Alikianou. The distance between the two populations is 350kms, two-thirds of this distance is sea. Crete (Alikianou) is on average $5^{o}C$ warmer and more dry all year round than the Parnes location. The two populations are genetically different (as observed by inversions from the 1964 sample (Krimbas, (1965)). The lethality and allelism of lethal at these populations are given in Table VIII (Panopoulou, 1973).

TABLE VIII

	Crete	Parnes
Frequency, lethals and semilethals	.277 ± .0034	.284 ± .004
Allelism frequency	.005 ± .0038	.0095 ± .0069
Isogenic chromosomes number	150	145

In D. subobscura chromosomal substitutions between strains are difficult because of the absence of suitable marker strains for more than two chromosomes. The only balanced strain which exists in this species, for isogenic lines, is the one which was earlier described for the O chromosome.

RESULTS

Table IX contains the chromosomal structure, the heat-sensitive phenotypes and electrophoretic alleles (mass extract technique) for the four loci for every isogenic line of the two samples.

Table X contains the frequencies of inversions and electrophoretic alleles estimated from Table IX in both samples.

(A) The Linkage-Disequilibrium Case

The results in Table IX are also gametic analysis so testing linkage equilibria or disequilibria is a very simple matter. Firstly, I shall discuss the linkage disequilibrium between the Xdh and Ao loci in the Parnes population and that because two years later (ten more generations) a new sample from the same location was analysed for inversions and enzymatic loci of O chromosome (Fountatou, Ph.D. Thesis, 1977; Loukas et al., in press, and personal communication).

The alleles which show linkage disequilibrium (Zouros and Krimbas, 1973) in both populations are the $\underline{Xdh}^{.94}$ and $\underline{Ao}^{.89}$ which correspond in my simpler notation to $\underline{Xdh}^{4}$ (4) and $\underline{Ao}^{4}$ (4) respectively. This gametic chromosomal combination was found significantly more frequently than random expectation in both samples. The probability according to the authors was .0015 by pure chance to observe more than ten gametes containing the combination in a sample of 115 examined chromosomes, determined by the allelic $\underline{Xdh}^{4}$, $\underline{Ao}^{4}$ frequencies in the Parnes sample.

According to the authors, the only chromosomal structures which include both loci are O_{3+4+1} and O_{3+4+22}, and more precisely the inversions O_1 and O_{22} (see Figure 2). From the eleven observed

TABLE IX. Chromosomal structure, heat-sensitive phenotypes (mass technique) and electrophoretic alleles of the tested isogenic strains.

CRETE

INVERSIONS	Xdh 72°C ± 0.5				Ao 72°C ± 0.5				Me 54°C ± 0.5				Odh 54°C ± 0.5			
	5'	10'	15'	allele	5'	10'	15'	allele	5'	10'	15'	allele	5'	10'	15'	alle
4. O_{3+4+8}	+	+	-	3	+	+	+	3	+	+	+	2	+	+	+	2
11 O_{3+4+8}	+	+	+	5	+	-	-	4	+	+	-	2	+	+	+	2
14 O_{3+4+22}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
15 O_{3+4+8}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
19 O_{3+4+8}	+	+	+	3	+	+	+	3	-	-	-	2	+	+	+	2
25 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
33 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
40 O_{3+4+8}	+	+	+	3	+	-	-	3	+	+	+	2	+	+	+	2
54 O_{3+4+8}	+	+	+	3	+	+	-	3	+	+	+	2	+	+	+	2
67 O_{3+4+8}	+	+	-	3	+	+	+	3	-	-	-	2	+	+	+	2
73 O_{3+4}	+	+	+	3	+	-	-	3	+	+	-	2	+	+	+	2
76 OST	-	-	-	Null	-	-	-	Null	+	-	-	2	-	-	-	2
81 O_{3+4+8}	+	+	+	3	+	+	+	3	-	-	-	3	+	+	+	2
85 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
92 O_{3+4+8}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
93 O_{3+4+8}	+	+	+	3	+	-	-	3	-	-	-	2	+	+	+	2
97 O_{3+4+1}	+	+	+	3	+	-	-	3	-	-	-	2	+	+	+	2
98 O_{3+4+8}	+	+	+	1	+	+	+	1	+	+	+	2	+	+	+	2
105 O_{3+4}	+	+	-	3	+	+	+	3	+	+	-	1	+	+	+	2
106 O_{3+4+8}	+	+	+	3/5	+	+	+	3/4	+	-	-	2	+	+	+	2
108 O_{3+4+8}	+	+	+	3	+	+	-	3	-	-	-	2	+	+	+	2
115 O_{3+4+1}	+	+	+	3/4	+	+	-	3	+	-	-	2	+	+	+	.2
116 O_{3+4+2}	+	+	+	3	+	+	+	3	-	-	-	2	+	+	+	2
118 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
120 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	lost			2
125 O_{3+4+8}	+	+	+	5	+	+	+	5	+	+	+	2	+	+	+	2
129 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
133 O_{3+4+8}	+	+	+	3	+	+	+	3	-	-	-	2	+	+	+	2
137 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
142 O_{3+4+8}	+	+	+	3	+	+	+	2	+	+	-	2	+	+	+	2
143 O_{3+4+8}	lost			4	+	+	+	3	+	+	+	2	+	+	+	2
146 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
151 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
153 O_{3+4+8}	lost			3	+	+	+	3	+	+	+	3	+	+	+	2
161 O_{3+4+8}	lost			3	+	+	+	3	lost				+	+	+	2

CONTINUED/

TABLE IX (continued) (2)

	Xdh				Ao.				Me:.				Odh			
165 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	
177 O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
179 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	lost			
198 O_{3+4+8}	+	+	-	3	+	+	+	3	+	-	-	2	+	+	+	
199 O_{3+4}	+	+	+	2	+	+	+	3	+	-	-	1	+	+	+	
201 O_{3+4}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	
202 O_{3+4+8}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	
203 O_{3+4+8}	+	+	-	3	+	+	+	3	+	+	+	2	+	+	+	
209 O_{3+4+8}	+	+	+	3	+	-	-	3	+	+	+	2	+	+	+	
210 O_{3+4}	lost			3	+	+	+	3	+	+	-	2	+	+	+	
218 O_{3+4+8}	+	+	+	2	+	+	+	2	+	+	-	2	+	+	+	
224 O_{3+4+7}	+	+	+	5	+	+	+	3	+	-	-	2	+	+	+	
227 O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
232 O_{3+4}	+	-	-	3	+	+	+	3	+	+	+	2	+	+	+	
237 O_{3+4+8}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	
238 O_{3+4+8}	+	+	+	3	+	+	+	2	+	+	+	2	+	+	+	
242 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	
247 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
259 O_{3+4+1}	+	+	-	3	+	+	+	3	+	-	-	2	+	+	+	
265 O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	
272 O_{3+4+8}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	
N	N = 56				N = 56				N = 56				N = 56			

CONTINUED (PARNES)/

TABLE IX (continued) (3)

PARNES

		Xdh				Ao				Me				Odh			
1	O_{3+4}	lost			4	+	+	+	3	+	+	−	2	+	+	+	
3	O_{3+4+1}	+	−	−	3	+	+	+	3	+	+	−	2	+	+	+	
6	O_{3+4+22}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
9	O_{3+4+22}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
10	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
12	O_{3+4+2}	+	+	+	3	+	+	+	3	+	+	−	2	+	+	+	
13	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
16	O_{3+4}	+	+	+	3	+	+	+	3	+	+	−	2	+	+	+	
14	O_{3+4}	+	+	−	3	+	+	+	3	+	+	+	2	+	−	−	
20	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
21	O_{3+4}	+	+	+	3	lost				lost				lost			
22	O_{3+4}	+	+	+	3	+	+	+	3	+	+	−	2	+	+	+	
24	O_{3+4+1}	+	+	+	3	+	+	+	3	+	+	−	2	+	+	+	
26	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
36	ST	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
40	ST	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
41	O_{3+4+1}	+	+	+	3	+	+	+	3	+	+	+	2	+	−	−	
43	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
46	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
50	O_{3+4}	lost			3	+	+	+	3	+	+	+	2	+	+	+	
52	O_{3+4}	+	+	+	3	+	+	+	3	+	−	−	2	+	+	+	
58	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
59	O_{3+4}	+	+	−	3	+	+	+	2	+	−	−	2	+	+	+	
60	O_{3+4}	+	+	+	1/3	+	+	+	3	−	−	−	2	+	+	+	
61	ST	+	+	+	3	+	+	+	3	+	+	−	2	+	+	+	
63	O_{3+4}	+	+	+	3	+	+	+	3	+	−	−	2	+	+	+	
64	O_{3+4}	+	+	+	3	+	+	+	3	+	−	−	2	+	+	+	
67	O_{3+4}	+	+	+	3	+	+	+	3	+	−	−	2	+	+	+	
69	O_{3+4}	+	+	−	1/3	+	+	+	3/2	+	−	−	2	+	+	+	
70	O6	+	+	+	3	+	+	+	3	+	+	−	2	+	+	+	
71	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
72	ST	lost				+	+	+	3	+	+	+	2	+	+	+	
73	O_{3+4+1}	+	+	+	3	+	+	+	3	+	+	−	2	+	+	+	
78	ST	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
79	ST	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
80	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	
81	O_{3+4+1}	+	+	+	3	+	+	+	3	+	+	−	1	+	+	+	

CONTINUED/

TABLE IX (continued)(4)

		Xdh				Ao				Me.				Odh			
82	O_{3+4}	+	+	+	1	+	+	+	2	+	+	+	2	+	+	+	2
83	O_{3+4+1}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
87	O_{3+4}	+	+	-	3	+	+	+	3	+	+	-	2	+	+	+	2
88	O_{3+4+2}	-	-	-	3	+	+	+	3	+	-	-	2	+	+	+	2
90	O_{3+4+22}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
92	O_{3+4}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
93	O_{3+4}		lost				lost				lost			+	+	+	2
94	O_{3+4}	+	+	+	3	+	+	-	3	+	+	+	2	+	+	+	2
96	ST	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
99	O_{3+4+2}	+	+	+	3	+	+	+	3	-	-	-	Null	+	+	+	2
102	O_{3+4}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
103	O_{3+4+22}	+	+	+	3	+	+	-	3	+	+	+	2	+	+	+	2
107	O_{3+4+22}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
108	O_{3+4}		lost		3	+	+	+	3	+	+	+	3	+	+	+	2
109	O_{3+4+2}	+	+	+	3	+	+	-	3	+	+	+	2	+	+	+	2
114	O_{3+4+1}	+	+	+	3	+	+	+	3	-	-	-	2	-	-	-	2
118	O_{3+4}	+	+	+	3	+	+	+	3	+	+	-	2		lost		
119	O_{3+4}	+	+	+	3	+	+	+	3	-	-	-	2	+	+	+	2
121	O_{3+4+1}	+	+	-	3	+	+	+	3	+	+	+	2	+	+	-	2
122	O_{3+4}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
123	O_{3+4}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
124	O_{3+4}	+	+	+	2	+	+	+	3	-	-	-	2	+	+	+	2
125	O_{3+4}	+	+	+	3	+	+	+	3	-	-	-	2	+	-	-	2
126	O_{3+4+22}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
132	O_{3+4+22}	+	+	+	3	+	+	-	3	+	-	-	2	+	+	+	2
138	O_{3+4+22}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
141	O_{3+4+1}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
142	O_{3+4}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
145	O_{3+4+1}	+	-	-	3		lost		3	+	+	-	2	+	+	-	2
146	O_{3+4}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
147	O_{3+4}	+	-	-	3	+	+	+	3	+	+	+	2	+	-	-	2
150	O_{3+4+8}	+	+	+	1	+	+	+	3	+	+	-	2	+	+	+	2
152	O_{3+4}	+	+	+	3	+	+	+	3	+	-	-	2	+	-	-	2
153	O_{3+4}	+	+	+	4	+	+	+	3	+	+	-	2	+	+	+	2
157	O_{3+4+7}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2

CONTINUED/

TABLE IX (continued) (5)

		Xdh				Ao				Me				Odh			
158	O_{3+4}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
159	O_{3+4+1}	-	-	-	3	+	+	+	3	+	-	-	2	-	-	-	2
160	O_{3+4+1}	+	+	+	3	+	+	+	3	+	+	-	2	+	+	+	2
161	O_{3+4+22}	+	+	+	3	+	+	+	3	+	-	-	2	+	+	+	2
162	O_{3+4}	+	+	-	3	+	+	+	3	+	+	+	2	+	+	-	2
192	O_{3+4+8}	+	+	+	3	+	+	+	3	+	+	+	2	+	+	+	2
N =					76				76				76				76

Comparison of nomenclature used to classify electrophoretic alleles by Zouros and Krimbas (1973), Tsakas (1976), and Loukas *et al.* (1978).

Zouros/Krimbas	Tsakas	Loukas *et al.*
	Xdh	
.88	0	0
.89	1	1
.90	2	2
.92	3	3
.94	4	4
.96	5	5
	Ao	
.82	0	0
.83	1	1
.85	2	2
.87	3	3
.89	4	4
.91	5	5

TABLE X. Frequencies of inversions, electrophoretic alleles in Crete and Parnes samples.

CRETE

Inversions Kind	Freq.	X_{dh} all.	freq.	Ao all.	freq.	Me all	freq.	O_{dh} all.	freq.
O_{ST}	.02	1	.02	1	.02	1	.04	1	.02
O_{3+4}	.04	2	.02	2	.04	2	.94	2	.98
O_{3+4+8}	.73	3	.83	3	.86	3	.02		
O_{3+4+1}	.05	4	.02	4	.02				
O_{3+4+2}	.02	5	.05	5	.02				
O_{3+4+22}	.02	Null	.02	Null	.02				
O_{3+4+7}	.02	3/5	.02	3/4	.02				
		3/4	.02						

PARNES

O_{ST}	.09	1	.03	1	0	1	.01	1	0
O_{3+4}	.54	2	.01	2	.03	2	.97	2	1.000
O_{3+4+8}	.03	3	.93	3	.97	3	.01		
O_{3+4+1}	.15	4	.03			Null	.01		
O_{3+4+2}	.05								
O_{3+4+7}	.01								
O_{3+4+22}	.12								
O_{6}	.01								

chromosomes ($\underline{Xdh}^4$ - $\underline{Ao}^4$) only one/was included in/the inversion O_{3+4+22} and none at the O_{3+4+1}, so the estimated linkage-disequilibrium was not a result of/an inversion including the two loci, a possibility not overlooked by the authors.

Table Xa contains the inversion (O chromosome) and allelic frequencies (Xdh and Ao) of Parnes population (same location) in three successive samples (1972, Zouros, Krimbas; 1974, our sample; 1976, Fountatou).

TABLE Xa

Inversions (O chromosome) and gene frequencies (Xdh and Ao) from the Parnes population in three successive samples (1972, 1974, 1976).

Inversion	1972	1974	1976
O_{ST}	.159	.09	.126
O_{3+4}	.424	.54	.526
O_{3+4+1}	.238	.15	.137
O_{3+4+2}	.007	.05	.042
O_{3+4+7}	.003	.01	0
O_{3+4+8}	.060	.03	.047
O_{3+4+22}	.079	.12	.111
Rest	0	.01	.011
Xdh			
$\underline{Xdh}^0$	.013	0	.005
$\underline{Xdh}^1$	.146	.03	.016
$\underline{Xdh}^2$	.099	.01	.142
$\underline{Xdh}^3$	.623	.93	.674
$\underline{Xdh}^4$	.093	.03	.116
$\underline{Xdh}^5$	.026	0	.037
$\underline{Xdh}^6$	0	0	.010

CONTINUED/

TABLE Xa (Continued)

	1972	1974	1976
$\underline{Ao}^{0}$	0	0	.016
$\underline{Ao}^{1}$	.013	0	.100
$\underline{Ao}^{2}$	.086	.03	.237
$\underline{Ao}^{3}$	.675	.97	.547
$\underline{Ao}^{4}$	.179	0	.053
$\underline{Ao}^{5}$	.047	0	.037
$\underline{Ao}^{6}$	0	0	0
N (number of chromosomes)	151	76	190
e.d.^{nm} $\underline{Xdh}^{4}$ - $\underline{Ao}^{4}$	yes	no	no*
generations approximately	0	10	20

* In order to assess the significance of the apparent differences between years the χ^2 test for homogenicity was applied. To obtain numbers of 5 or more, it was necessary, for both the Ao and Xdh loci, to pool the 'slow' alleles 4, 5, 6 and the 'fast' alleles, 0, 1, 2 and to compare both totals with the frequency of the common allele 3. The results of the tests showed: (1) no statistically significant differences in inversion frequencies among the three years or between any two of them, but (2) there were statistically significant differences in the Xdh and Ao allele frequencies among the three years and any combination of two of them.

From Table Xa the following conclusions can be drawn:

(1) The inversion frequencies do change somewhat.

(2) The gene frequencies vary to a greater degree.

(3) If the observed linkage disequilibrium in the 1972 sample was the result of epistatic natural selection, we should observe it (supposing that the direction or intensity of selection did not change in ten or twenty generation intervals) again in the 1974 sample, or at least in the large sample of 1976.

(4) The frequencies of the $\underline{Xdh}^4$ allele in the three successive samples are .093, .03, .116 and of $\underline{Ao}^4$ .179, 0, .053.

(5) According to the earlier observation and without counting the small intermediate sample, the gene frequencies (of the favoured gametic combination $\underline{Xdh}^4$ - $\underline{Ao}^4$) for the $\underline{Xdh}^4$ is stable while the $\underline{Ao}^4$ fell by a factor of three.

(6) The sum of the frequencies of the two inversions O_{3+4+1} and O_{3+4+22} (which contain the two loci) changed somewhat while the gene frequencies changed to a greater extent (see below).

	1972 (N = 131)	1974 (74)	1976 (190)
$\underline{Xdh}^4$	.093	.03	.116
$\underline{Ao}^4$	.179	0	.053
$\underline{Xdh}^4$ - $\underline{Ao}^4$	exp. 1 obs. 11	exp. 0 obs. 0	exp. 1 obs. 1
O_{3+4+1} + O_{3+4+22}	.317	.27	.24

(7) It appears that the linkage disequilibrium observed in the 1972 sample of the Parnes population was due to sampling or drift and not as a result of epistatic natural selection.

The hidden heat-sensitive polymorphism is of no use because as we shall see later this polymorphism seems to be the result of an interaction between different enzymatic products and not polymorphism of the structural loci. The results of the Crete samples in 1972 and 1974 are given in Table Xb.

Table Xb.

Inversions (O chromosome) and gene frequencies (Xdh and Ao) from the Crete population in two successive samples (1972, 1974).

	1972	1974
Inversions		
O_{3+4+8}	.817	.73
O_{3+4}	.099	.14
O_{ST}	.038	.02
O_{3+4+1}	.038	.05
O_{3+4+22}	.008) .046	.02) .07
rest	0	.02
Xdh		
Xdh^0	.069	0
Xdh^1	.015	.02
Xdh^2	.031	.02
Xdh^3	.809	.83
Xdh^4	.061) .87	.02) .85
Xdh^5	.015	.05
Null	0	.02
rest 3/5,3/4	0	.04
Ao		
Ao^0	.008	0
Ao^1	.023	.02
Ao^2	.023	.04
Ao^3	.748	.86
Ao^4	.145) .893	.02) .88
Ao^5	.053	.02
Null	0	.02
rest 3/4	0	.02
number of tested chromosomes	131	56

	1972	1974
O_{3+4+1}, O_{3+4+22}	.046	.07
Xdh^4	.061	.02
Ao^4	.145	.02
Xdh^4 - Ao^4	exp. 1 obs. 6	exp. 0 obs. 0

The χ^2 homogeneity test was applied (as in the Parnes case) and showed homogeneity between the two samples in inversions and electrophoretic alleles of the loci Xdh and Ao. From this Table we can conclude the following:

(1) Between the two samples the frequencies of inversions and Xdh and Ao alleles do change a little with the exception of the frequencies of the Ao^4 and Ao^3 (common) alleles.

(2) The frequency of the Ao^4 allele decreases from .145 in the 1972 sample to .02 in the 1974 sample.

(3) If the linkage-disequilibrium observed in the 1972 sample was the result of epistatic natural selection, we must observe a higher frequency in the chromosomal combination Xdh^4 - Ao^4, or at least in the allelic frequencies of Xdh^4 and Ao^4 in the 1974 sample. It is obvious from Table Xb that we did not observe any chromosome of the favoured combination Xdh^4 - Ao^4 as expected, and the frequencies of Xdh^4 decreased from .061 to .02; the same is true for the Ao^4 gene frequency which decreased from .145 to .02.

(4) It, therefore, appears that the linkage-disequilibrium observed in the 1972 sample was a result of sampling or genetic drift (as we shall discuss later) and not a result of natural selection.

In general, therefore, by this simple analysis with relatively small samples and by using three successive samples from the Parnes location (1972, 1974 and 1976) and two from the Crete population (1972 and 1974), the linkage-disequilibria observed in the 1972 samples among the Xdh and Ao as a favourite combination Xdh^4 - Ao^4 was not the result of natural selection but of sampling or genetic drift.

Despite the small number of samples I will apply a 2 x 2 table of gametic combinations for the loci Xdh and Ao (common allele compared to the rest) to see whether there is any tendency towards co-occurence of the alleles in the two samples.

CRETE

	Xdh^3 obs.	Xdh^3 exp.	rest obs.	rest exp.	
Ao^3	44	(40)	3	(7)	47
Ao rest	2	(6)	5	(1)	7
	46		8		54

PARNES

	Xdh^3 obs.	Xdh^3 exp.	rest obs.	rest exp.	
Ao^3	68	(67)	1	(2)	69
Ao rest	4	(5)	1	(0)	5
	72		2		74

These estimates do not show any apparent tendency towards co-occurence in the Parnes sample, while in the Crete sample the common alleles of the two loci are combined more frequently and so are the rare. The Fisher exact test applied to the Crete sample of fifty-four examined chromosomes gave a probability of .000334 for this or more extreme discrepancies. The data are presented in Table XI.

TABLE XI.

CRETE

alleles Ao	3	3	3	3	2	2	1	4	5	Null	
alleles Xdh	3	5	4	2	3	2	1	5	5	Null	
No. of chromosomes	44	1	1	1	2	1	1	1	1	1	54

This may be regarded as evidence that common alleles and rare alleles of the two loci are combined together more frequently in this sample, and that this is not due to the material which we

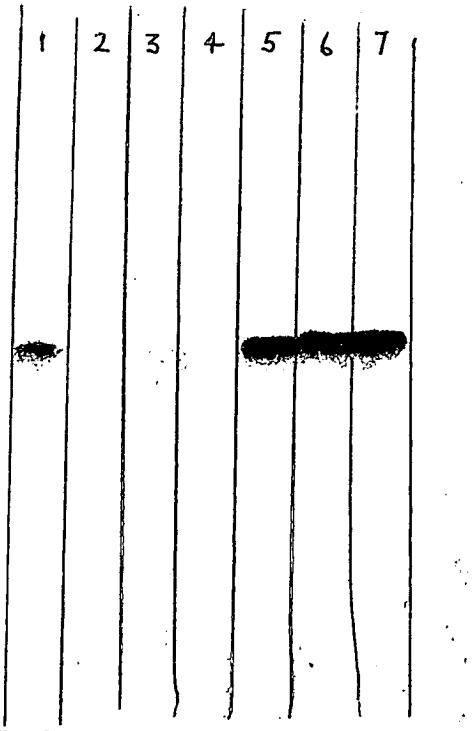
use for trapping as there is not a specific gametic combination attracted. In another case reported by Krimbas and Alevizos (1973), the same type of result was observed by using inversions as genetic markers (see Conclusions). This must be due to the local genetic microdifferentiation at that time. If it is true that local microdifferentiation does exist in D. subobscura populations this may be a reason for linkage disequilibrium due not to genetic drift of the population as a whole but to local genetic drifts or microdifferentiation. The case investigated by Zouros and Krimbas (1973) seems to be of this kind, since the inversion frequencies in both populations over the 1963-1972 period (Tables V, Xa, Xb), and the 1974-1976 period (Tables X, Xa), change less than would be expected on the basis of genetic drift.

Useful observed isogenic strains (Table IX).

The strain with number 99 from the Parnes sample is a null isogenic strain for Me locus. The isogenic lines with numbers 106 and 115 from the Crete sample both produced two electrophoretic variants of Xdh and the former line produced two variants of Ao as well. The isogenic lines with numbers 60 and 69 from the Parnes sample both had two active alleles of Xdh and the latter had two of Ao.

Line number 76 in the Crete sample has null alleles for the Ao and Xdh loci, while Me has an active allele and is located between the two. The only aldehyde-oxidase activity which we can investigate electrophoretically in D. subobscura is produced by the locus Ao (map position 55.9). The only xanthine-dehydrogenase activity which we can investigate electrophoretically in this fly

is produced by the locus Xdh (map position 87.3). The phenotype of the Xdh null strain seems to be a "rosy" one but we did not see any activation in vivo or in vitro after using molybdenum concentrations (E. Panopoulou and S. Tsakas, 1978) as Glassman and Mitchell (1959) described. On the contrary, activation appeared for active alleles (see Photograph 2).



Photograph 2. Xdh: from the left to the right 1 common (3) allele. 2,3,4 null alleles. 5 common (3) alleles after Mo^{++} feeding. 6, 7 allele (2) after Mo^{++} feeding.

It is the first time that either null alleles or chromosomes carrying two active alleles have been reported at these loci and in these populations. The evidence supporting duplication in the strain 106 for both the Xdh and Ao loci is as follows: (1) Sixteen adult flies were individually analysed and all showed enzyme bands corresponding to both Xdh^3 and Xdh^5 and to both Ao^3 and Ao^4. (2) The same results were observed when sixteen offspring from a single cross between a virgin female from the strain 106 and a male from the null strain 76 were analysed. We do not have estimates of the frequencies of these events in the past. This can be a reason for biased estimations (in Xdh, Ao and Me) of active allele frequencies when the strain used for crossing carries active alleles, as in the Zouros and Krimbas case

the ch, cu (cherry, curled) strain which is homozygote for the common alleles Xdh, Ao and Me. (Xdh^3, Ao^3 and Me^2).

(B) The heat-sensitive polymorphism case

Thus far I have described the linkage disequilibria using only the electrophoretic and inversions polymorphisms and have not used any of the observed heat-sensitive variation (Table IX) which was one of the main purposes of this study (see final paragraph of Introduction).

The reason for not using the heat-sensitive "alleles" was that in my case I had evidence that the variation was not caused only by the structural loci but probably was the result of interaction among the enzymatic products of different loci.

Table IX in addition to presenting the electrophoretic and inversions polymorphism also presents the new hidden heat-sensitive variation observed by analyzing with mass extract technique one sample from each isogenic strain for the loci Ao, Me and Xdh, and Table XII presents a summary of this.

In order to test whether the observed heat-sensitive alleles were hidden alleles of the tested loci Ao, Me and Xdh, I decided to apply the arguments of the authors listed at the bottom of page 9.

Their arguments using mass extract techniques were:

(a) Repeatability within the tested strains.

(b) Homogeneous heat-sensitive patterns within F_1 crosses.

(c) One locus segregation results using F_2 crosses.

Note that as pointed out in the Introduction, there is a contradiction between the results of Trippa et al. (1976) and Cochrane (1976).

TABLE XII

"Hidden" new alleles which were revealed after mass extract heat-sensitive treatment of the two samples and ratio heat-sensitive to electrophoretic alleles. Simpler representation of the results from Table IX.

(A) CRETE

Hidden alleles: Xdh	freq.	Ao	freq.	Me	freq.	Odh	freq
Null (1)	.02	Null	.02				
				Me^1+++ (1)	.02	Odh^1+++ (1)	.02
Xdh^1++ (1)	.02	Ao^1+++ (1)	.02	Me^1+-- (1)	.02	Odh^2+++ (52)	.96
				Me^2+++ (22)	.40	Odh^2--- (1)	.02
Xdh^2+++ (2)	.04	Ao^2+++ (3)	.05	Me^2++- (10)	.19		
Xdh^3+++ (37)	.74	Ao^3+++ (40)	.73	Me^2+-- (12)	.22		
Xdh^3++- (6)	.12	Ao^3++- (3)	.05	Me^2--- (6)	.11		
Xdh^3+-- (1)	.02	Ao^3+-- (5)	.09				
				Me^3+++ (1)	.02		
		Ao^4+-- (1)	.02	Me^3--- (1)	.02		
Xdh^5+++ (2)	.04	Ao^5+++ (1)	.02				
	7		8		8		3
*7/5 = 1.4		*8/6 = 1.3		*8/3 = 2.7		*3/2 = 1.5	

(B) PARNES

Xdh^1+++ (2)	.03						
				Null (1)	.01		
Xdh^2+++ (1)	.01	Ao^2+++ (2)	.03	Me^1++- (1)	.01		
Xdh^3+++ (57)	.81	Ao^3+++ (68)	.92	Me^2+++ (30)	.41	Odh^2+++ (65)	.88
Xdh^3++- (5)	.07	Ao^3++- (4)	.05	Me^2++- (20)	.27	Odh^2++- (3)	.04
Xdh^3+-- (3)	.04			Me^2+-- (16)	.22	Odh^2+-- (4)	.05
Xdh^3--- (2)	.03			Me^2--- (5)	.07	Odh^2--- (2)	.03
Xdh^4+++ (1)	.01			Me^3+++ (1)	.01		
	7		3		7		
*7/4 = 1.75		*3/2 = 1.5		*7/4 = 1.75		*4/1 = 4.00	

* = ratio hidden over electrophoretic alleles

TABLE XIII

Tested strains with and without repeatability for the loci Ao , Me and Xdh. For the strains with repeatability I applied the single fly-technique (s.f.t.) to check if this gives the same heat-sensitive phenotypes as the mass technique in strains with repeatability.

Ao

Strain without repeatability	alleles	heat-sensitive phenotypes	replicates
P_{94} ++–	3	+++, +– , ++–	3
K_{238} +++	2	+– ,+–, ++–	3
P_{82} +++	2	++–, +++, ++–	3
Strain with repeatability			
K_{40} +–	3	+–	4
		s.f.t. +–	4
K_{98} +++	1	+++	4
		s.f.t. +++	5

Me

Strain without repeatability			
K_{93} ---	2	+–, +–	2
K_{153} +++	3	++–, +++	2
P_{81} ++–	1	+–, +–	2
Strain with repeatability			
P_{192} +++	2	+++	3
		s.f.t. +++	5
K_{73} ---	2	---	4
		s.f.t. ---	4

Xdh

Strain without repeatability			
P_{153} +++	4	+–, ++–, +–	3
P_{59} ++–	3	+++, +++	2
P_{147} +–	3	++–, +++, +–	3
Strain with repeatability			
P_{82} +++	1	+++	3
		s.f.t. +++	3
K_{203} ++–	3	++–	2
		s.f.t. ++–	4

My strains were at least isogenic for the segment II on which the tested loci Ao, Me, Xdh and the mutations cu and Ba of the balanced used strain are located (see Figure 2). Therefore their arguments had to apply in my case for every tested strain.

(a) Testing the repeatability of heat-sensitive alleles within tested strains.

Due to the amount of time required for testing all the strains I randomly selected five strains for every locus and I analyzed more samples within these strains. For each locus I found strains with (two) and without (three) repeatability (Table XIII).

It was not expected that some strains would show non-repeatable phenotypes.

In order to test whether the non-repeatable phenotypes were due to technical factors or to genetic variation within the strain, I carried out further experiments. For example, as presented in Table XIII, the strain K_{238} showed three different heat sensitive phenotypes when from each sample of twelve flies a separate supernatant was produced and analysed. However when forty-eight flies from the same strain were homogenized to produce one major supernatant and this was then divided into four samples and run in different gels, these samples showed only one heat sensitive phenotype. This meant that the electrophoretic technique was standard. The different factor in these two cases seems to be that in the first case four different supernatants were analysed while in the second, the same one was analysed four times. So, it seems that the four different supernatants were different in composition, either because of a significant genetic difference between different random samples of twelve flies from the same strain, or because of variations in the extraction procedure. It is important to mention here

that the results were the same when I applied this procedure to other non-repeatable strains and also to the offspring of F_1 crosses between two strains one or both of which were non-repeatable. On the other hand, when this procedure was applied to repeatable strains the same phenotype characterizing the strain was observed.

Since I performed these experiments, Singh et al. (1976) have observed similar lack of repeatability between samples of six adult flies from isogenic lines of Drosophila pseudobscura.

At this point I wanted to test whether or not my single-fly technique would give the same heat-sensitive alleles as the mass extract technique. I wanted to know if I could use this technique for single offspring analysis which is a fundamental necessity for one locus Mendelian segregation. The only strains suitable for this were the strains with repeatability (Table XIII). The results of this experiment are presented in the same Table XIII and it is obvious that the single fly technique is giving the same heat-sensitive phenotypes as the mass extract technique.

(b) Testing homogeneity in samples within F_1 crosses.

In order to gather further information, I performed mass F_1 crosses using some strains without repeatability from the Table XIII to test whether within the F_1s I would observe, by using the mass technique, one type of heat-sensitive pattern.

In these F_1 crosses the strains designated by a star had not been repeatable (Table XIII). The phenotypes of these strains were estimated by analysing the parents. In each cross the first strain presented are the female parents. These results, locus by locus, are presented below.

Ao

1st cross

$$*P_{94}(3{+}{+}{-}) \times *P_{82}(2{+}{+}{+})$$

Three F_1 samples gave me the following phenotypes:

$$\frac{2{+}{+}{+}}{3{+}{+}{+}}, \quad \frac{2{-}{-}{-}}{3{-}{-}{-}}, \quad \frac{2{-}{-}{-}}{3{-}{-}{-}}$$

2nd cross

$$*K_{238}(2+--) \times K_{40}(3+--)$$

Four F_1 samples gave me the following phenotypes:

$$\frac{2+--}{3+--}, \quad \frac{2+--}{3+--}, \quad \frac{2++-}{3++-}, \quad \frac{2+++}{3+++}$$

3rd cross

$$*K_{238}(2+--) \times K_{11}(4+--)$$

Three F_1 samples gave me the following phenotypes:

$$\frac{2+--}{4+--}, \quad \frac{2++-}{4++-}, \quad \frac{2++-}{4++-}$$

In addition I present here another observation - the negative correlation between temperature and heat-sensitivity in the strain K_{98}. The single fly technique showed for the isogenic strain K_{98} (Table XIII), (1+++) heat-sensitive phenotype at 72°C.

I accidentally forgot to increase the temperature in the oil-bath from 54°C to 72°C and the samples (single flies) were for Ao detection. One fly was from the strain K_{98} which showed an unexpected heat-sensitive phenotype 1+-- instead of the repeatable 1+++. I repeated this test four times and in all cases I observed the same heat-sensitive form 1+--. One possible explanation for this surprise result is that another factor (e.g. an enzyme) is acting on the Ao products at 54°C, but not at 72°C.

Me

1st cross

$$*K_{153}(3+++) \times *K_{93}(2+--)$$

Eleven F_1 samples showed the following phenotypes:

$$\frac{2+++}{3+++}\ (2), \quad \frac{2++-}{3++-}\ (2), \quad \frac{2+--}{3+--}\ (4), \quad \frac{2---}{3---}\ (3).$$

2nd cross

$$P_{192}(2+++) \times *K_{81}(1+--)$$

Three F_1 samples showed the following phenotypes:

$$\frac{1+--}{2+--}, \quad \frac{1+++}{2+++}, \quad \frac{1+++}{2+++}$$

Xdh

1st cross

$$*P_{153}(4+--) \times *P_{59}(3+++)$$

Seven F_1 samples showed the following phenotypes:

$$\frac{3+++}{4+++}\ (5), \quad \frac{3++-}{4++-}\ (2)$$

2nd cross

$$P_{82}(1+++) \times *P_{147}(3+++)$$

Three F_1 samples showed the following phenotypes:

$$\frac{1+--}{3+--}, \quad \frac{1+--}{3+--}, \quad \frac{1+++}{3+++}$$

3rd cross

$$*P_{153}(4+++) \times *P_{147}(3++-)$$

Three F_1 samples showed the following phenotypes:

$$\frac{3+--}{4+--}, \quad \frac{3++-}{4++-}, \quad \frac{3+++}{4+++}$$

4th cross

$$K_{203}(3+--) \times K_{11}(5+++)$$

Five F_1 samples showed the following phenotypes:

$$\frac{3+++}{5+++}\ (5)$$

In addition in the Xdh case I want to present here (because of the presented Photograph 3) the results of a single cross and single fly technique applied: 1♂(3+--) x 1♀(3+++)

Photograph 3. Xdh 5th cross from right to the left.

parent ♀-offsp. -offsp. -offsp. -offsp. - parent ♂

3+-- 3--- 3--- 3++- 3+-- 3+++

In F_1 I analyzed eight single offspring (s.f.t.) and I got the following heat-sensitive phenotypes:

(3---) 3, (3+--) 3, (3++-) 2.

(c) Segregation analysis using test-crosses

After the above indications I decided to see whether or not the heat-sensitive phenotypes of the tested loci were the results of the action of the products of a single locus. The results of this experiment are presented locus by locus below.

Ao.

One female offspring from the 3rd F_1 cross ($*K_{238}(2+--)$ x $K_{11}(4+--)$) was mated with one male from the null strain K_{76} (Tsakas,

1978) as follows:

$$1♀ \frac{2+--\ O_{3+4+8}}{4+--\ O_{3+4+8}} \times 1♂ \frac{null\ O_{ST}}{null\ O_{ST}}$$

Using the single fly technique I analyzed sixty-two offspring and I observed the following four (instead of two) categories of electrophoretic, heat-sensitive phenotypes.

(2---) 15
(2+--) 12
(4---) 18
(4+--) 17

A ratio of 1:1:1:1 occurred between electrophoretic and heat sensitive phenotypes. Two hypotheses can explain this result. One is random variation, which can apply to all the following crosses; the other is that a genetic factor unlinked to the Ao structural locus is controlling the heat sensitive phenotypic expression of the alleles 2 and 4. This factor has two alleles, dominant resistant and recessive sensitive. This explanation could fit the above results if one parent is heterozygous and the other homozygous for the recessive.

Me

One female offspring from the 2nd F_1 cross $P_{192}(2+++) \times *K_{81}(1+--)$ was mated with one male from the null strain P_{99} (Table XII) as follows:

$$1♀ \frac{1+--\ O_{3+4+8}}{2+--\ O_{3+4+8}} \times 1♂ \frac{null\ O_{3+4+2}}{null\ O_{3+4+2}}$$

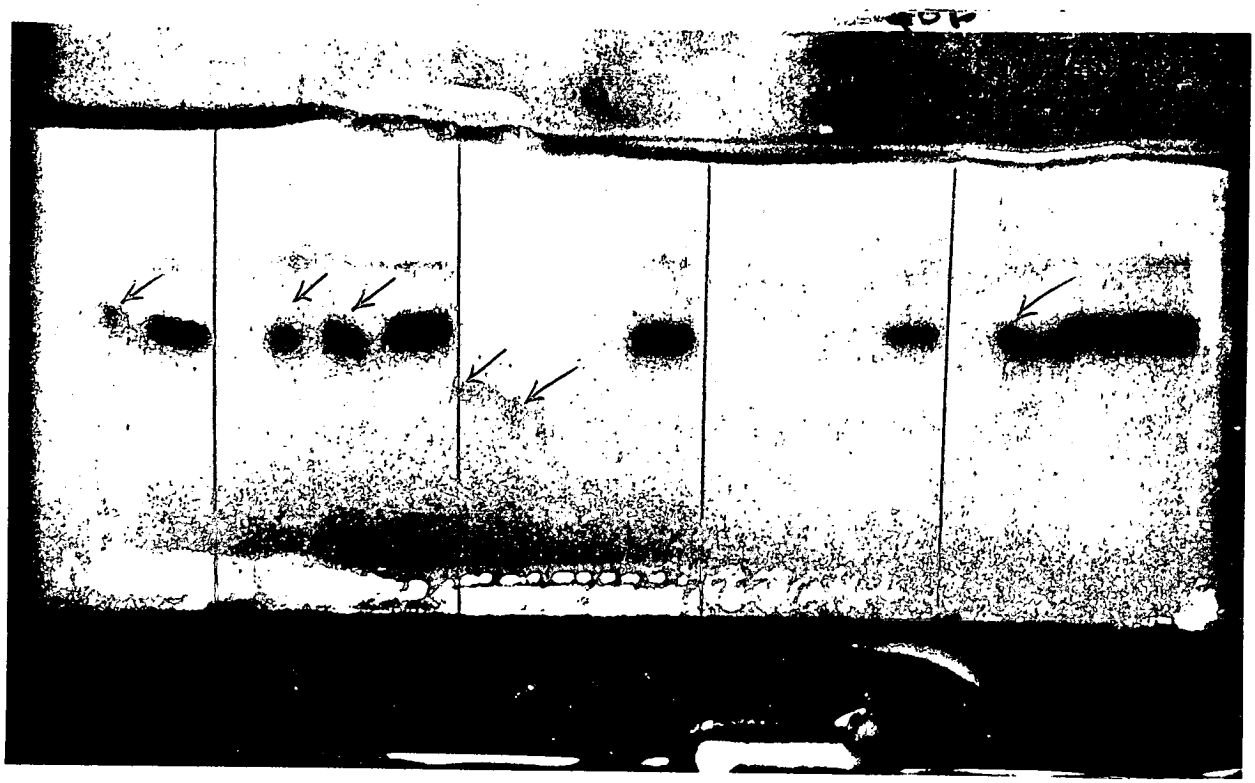
I analyzed six offspring and I observed the following phenotypes:

(2---) 3
(1---) 1
(2+--) 1
(1+--) 1

Since the offspring of this cross must have either a $\frac{1}{null}$ or a $\frac{2}{null}$ genotype, the same electrophoretic heat sensitive pattern must be observed

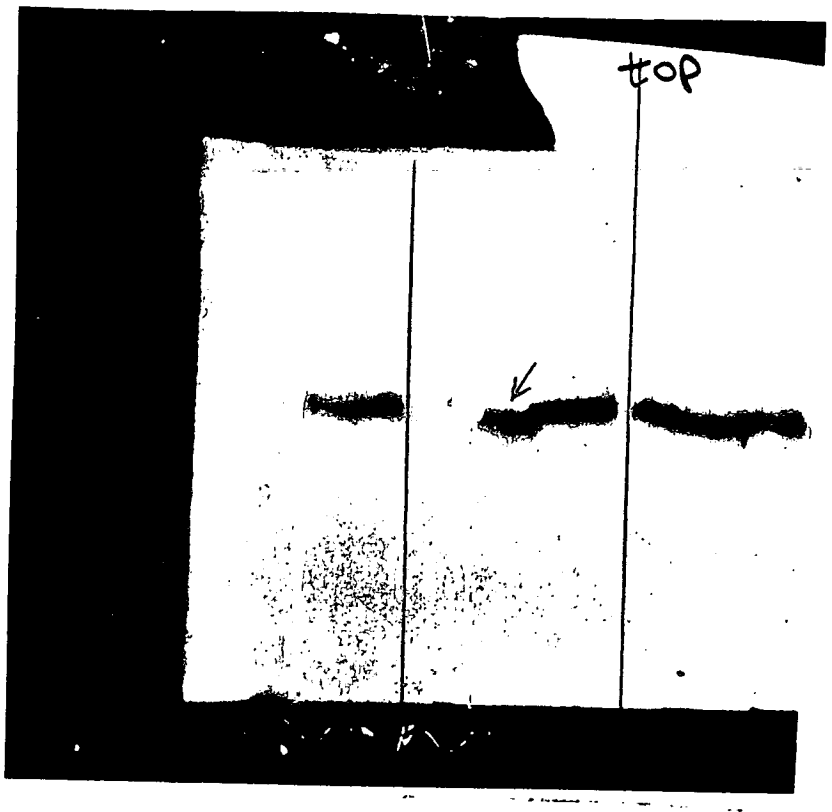
within each genotypic category. These results are evidence that the factor(s) determining the heat sensitive phenotype is not solely controlled by the structural Me locus.

In addition, I observed some rather irregularly-shaped zones of enzyme activity on the gel (see Photograph 4), and this was the reason that I did not analyse more offspring. These could have been experimental artifacts unrelated to the normal enzyme. A speculation which also occurred to me was that the enzyme had been partially disaggregated by peptidase action. This occurred to me because the zones varied in number and/or position within one analysed individual or between analysed individuals possessing the same genotype.



Photograph 4. Me test cross from right to the left: 1+--, 2---, 2---, 1---, 2---. This photograph was exposed longer than usual in an effort to more clearly show the faint irregularly-shaped zones of enzyme activity. So, these bands appear more distinct than they were on the gel. The faint blotches and those not in line with the marker were read as minus.

Irregularly shaped zones of enzyme activity also appeared when the mass technique was applied (see Photograph 5).



Photograph 5: Me:(mass extract technique) from right to left. 2+++, 2+ "peptides" -, 2+--. ↙ indicates "peptides".

Xdh

One female offspring from the 3rd F_1 cross ($*P_{153}$(4+++) x $*P_{147}$(3++-) was mated with one male from the null strain K_{76} (Tsakas, 1978) as follows:

$$1♀ \frac{3++- \quad O_{3+4}}{4++- \quad O_{3+4}} \times 1♂ \frac{\text{null } O_{ST}}{\text{null } O_{ST}}$$

I analyzed eighteen offspring and I observed the following phenotypes:

(3+--)	6
(4+--)	7
(3+++)	2
(4+++)	1

(3---) 1

(4---) 1

It is obvious that in this case the heat-sensitive phenotypes are not an expression of the structural Xdh locus only (alleles 3 and 4).

In summary I did not observe, utilizing my methods and materials, any clear evidence for the tested loci and alleles that the observed heat-sensitive polymorphism was due to "hidden" alleles of the structural examined locus. On the contrary, it seems that the heat-sensitive polymorphism is the result of interaction between the enzymatic products of different loci (see Conclusions).

CONCLUSIONS

(A) Linkage disequilibria

The linkage disequilibrium observed in 1972 samples (Zouros and Krimbas, 1973) appeared to be the result of sampling or genetic drift and not a result of epistatic natural selection.

Evidences for this are:

(a) I did not observe any increase in frequencies of the favoured combination, $\underline{Xdh}^4$-$\underline{Ao}^4$, neither in the alleles $\underline{Xdh}^4$, $\underline{Ao}^4$, after a time of ten generations interval in both populations.

(b) In the Parnes population and after ten more generations, Loukas et al. (1978) too, did not observe any linkage disequilibrium.

(c) In the case of the Crete sample it seems that local genetic microdifferentiation (loci Xdh and Ao) existed at the sampling period. This microdifferentiation can be due to the biological habits of the fly under natural conditions. The D. subobscura species uses yeast, fungus, rotten fruits and leaves as sources of food (larvae stages) and as a substrate for laying eggs (see Smart, 1945; Buzzati - Traverso, 1948; Basden, 1952). These sources exist as a mosaic in the population area. The species is monogamic (Maynard Smith, 1975). The pregnant female lays many eggs (probably full sibs) within a short period of time under food supply, so that a lot of full-sibs begin their lives simultaneously under the same environmental conditions; the majority of these will emerge simultaneously.

These emerging adult progeny of a single pair will probably remain close utilizing the food source and they will be able to mate within a few hours. If this does occur then trapping with a few traps in these phases can be a reason for genetic heterogeneity among

the traps even twenty metres apart as Krimbas and Alevizos (1973) observed, or for sampling producing local linkage disequilibrium.

(d) At both loci I found null alleles and probably also chromosomes carrying two structural genes. This can bias the exact estimations of allele frequencies when the strain used for crossing the wild males carries active alleles.

(e) Under lab conditions, Tsakas (1978) observed selection after ethanol (in food) treatment against the null strain (for Ao and Xdh) K76 (death in the first instar larval stage) in comparison to the strain K106 (carrying two copies chromosome for Ao and Xdh).

(B) Heat-sensitive polymorphism

In general the heat-sensitive mass technique gave non-repeatable results for the Xdh, Ao' and Me loci. The new single fly technique allows analysis of individual flies and thus Mendelian segregation may be studied by examining single zygotes.

Now we shall discuss the results of each tested locus.

Aldehyde oxidase

Lack of repeatability of heat sensitive phenotype was observed in some tested strains (see Table XIII) of this locus. When F_1 individuals resulting from a cross between two strains were analysed the phenotypes were again not repeatable (samples of twelve females were used in the main experiments). Segregation in test cross and single fly analyses showed that the heat-sensitive-electrophoretic phenotypes may under the control of another genetic factor unlinked to the structural Ao locus. It is difficult to explain why when treated with a lower temperature, more heat-sensitivity was observed in strain K98. It is possible that another factor is operating at 54°C (probably enzymatic) which is inactive at 74°C.

Malic enzyme

In some strains or in F_1 crosses between the strains, the heat-sensitive phenotype was not repeatable (see Table XII Segregation analysis in test cross and single fly analysis gave no one locus Mendelian segregation. I did not analyse more progeny as in the Ao case because I observed peptides with M.E. activity on the gel. I shall now discuss a possible hypothesis for this occurrence and some experimental evidence which supports it. If the bands which I observed were peptides this could be a result of peptidase action. These peptidases should have a higher concentration in the digestive system of the adult flies. If this is true, strains with a very sensitive repeatable phenotype (like the K73, Table XIII) should give a more heat resistant phenotype when I treat single flies with the digestive systems removed.

I therefore ran in one gel six adults (K73) from which the digestive systems had been removed (as well as I could dissect them out

using the microscope) and I read in all six more heat-resistant phenotypes, as follows:

++-, +--, ++-, ++-, +--, +--

In another set of six flies I removed only the stomachs (the stomach is very easy to remove by gently pulling out the head, but it is very difficult to homogenize it). In this case I observed again six out of six more heat resistant forms as follows:

++-, ++-, +--, +--, ++-, ++-

From these results it may be hypothesised that:

(1) the action of peptidase may have been observed on the gel, and

(2) this peptidase may be mainly located in the digestive system of the fly.

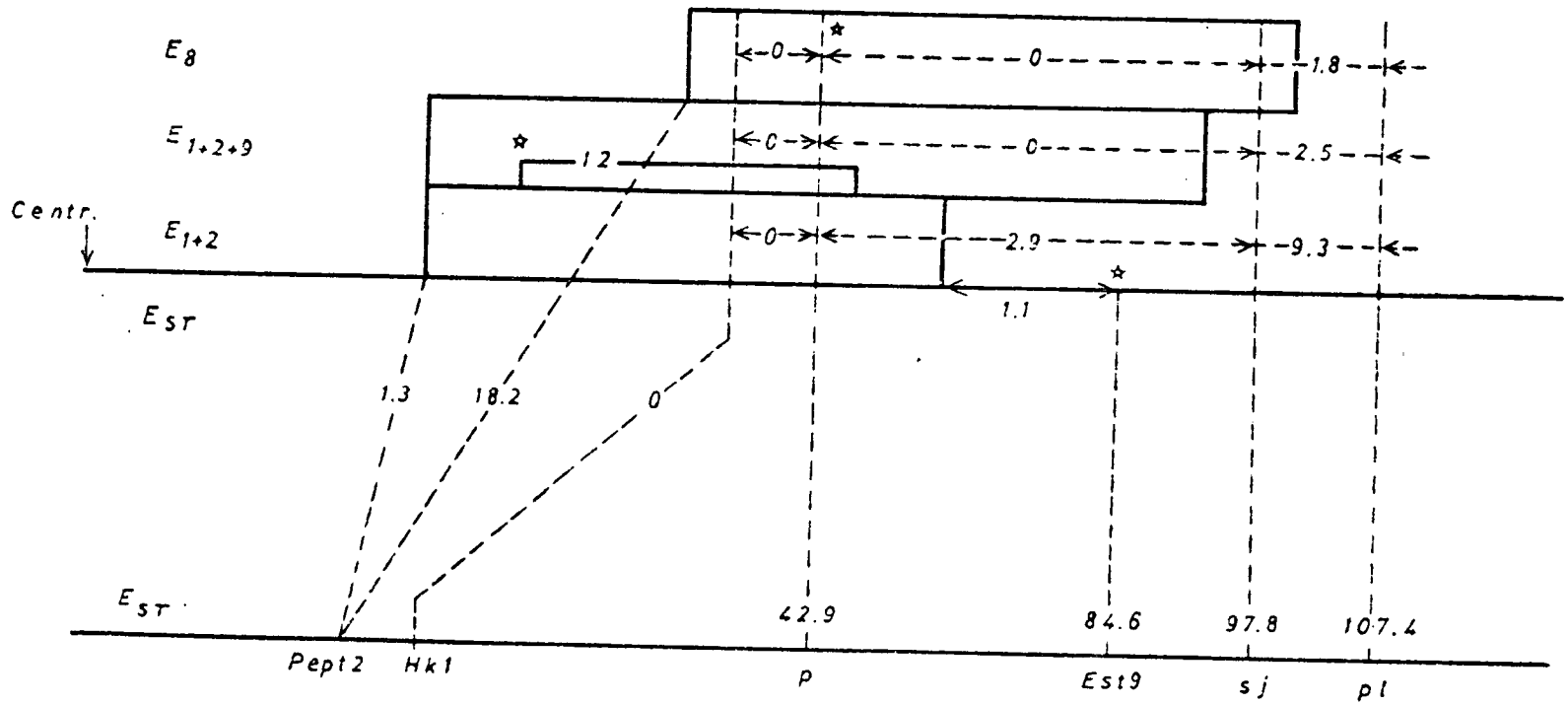


Figure 3. Cytological and genetical map of E Chromosome (from Loukas et al., 1978).

If these deductions are true then by homogenizing even a single fly and heating its crude extract we bring together at least two enzymes; the first of which (probably a peptidase) uses as substrate the second (M.E.) or any other enzyme present in the extract.

At this point I must answer the next question which will naturally occur to the reader - why were not peptides observed in the other two enzymes examined? A possible explanation is that the observation of peptides depends not only upon the peptidase action but also on the properties of the enzyme which is used as substrate (for example, the degree to which it is allosteric).

Is anything known about the genetic control of the peptidase enzyme(s) in this species? (Loukas et al., 1978) / In D. subobscura, three peptidase loci have been described to date, unlinked to our examined segment. They are:

(a) Lap (leucine-amino peptidase) located on the O chromosome as map 1 indicates. It has eight alleles (1.25, 1.18, 1.11, 1.06, 1.00, 0.92, 0.86 and 0.69) the heterozygotes appear without hybrid zone on gels. It is active during all stages of the life cycle of the fly.

(b) $pept_1$ (peptidase 1), located on the O chromosome, has three active alleles (1.60, 1.00 and 0.40) and a non-active (null) one. The heterozygotes have a hybrid zone. It is also active during all the life cycle of the fly.

(c) $pept_2$ (peptidase 2) located on the E chromosome (map 3), has four active alleles (1.08, 1.00, 0.92 and 0.83), the heterozygotes with hybrid zone, and is active at all stages of the life cycle.

Leucine aminopeptidase differs in its specificity from the other two peptidases, and we used different substrates in the staining reaction. When we stained specifically for Lap the other two peptidases did not cross-react. The reverse is also true (see reactions 1 and 2)(following page).

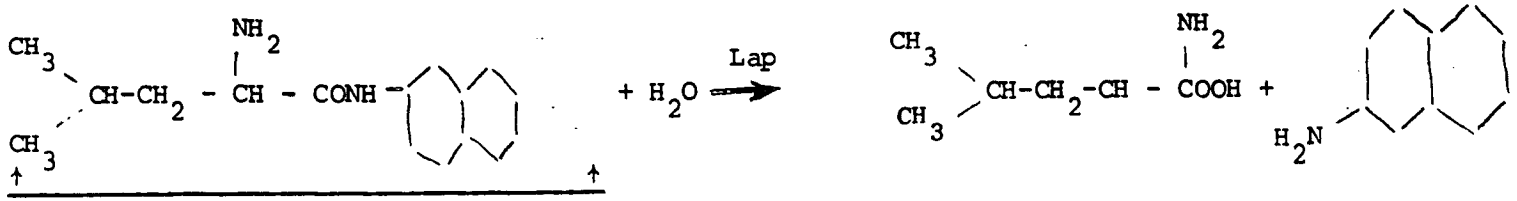
Activities within the loci (Alleles) have not yet been compared. One important point is the existence of the null allele at the $pept_1$ locus. This existence can be a reason for quantitative differences among peptidase genotypes. There is no estimation of the frequency of the null allele in any natural population yet. The Lap and $pept_1$ loci are both located in the O_{3+4} and O_{3+4+8} inversions (see Figure 2).

From John Altiparmakis' (Ph.D. thesis, Athens, 1975), experimental work, the gene frequencies between Crete and Parnes populations are not different (homogeneity of $\hat{F}$ values). He tested twelve loci with thirty-six alleles, although unfortunately the two peptidases(1 and 2) loci were not included in this study, probably their own allelic frequencies must be similar in the two populations.

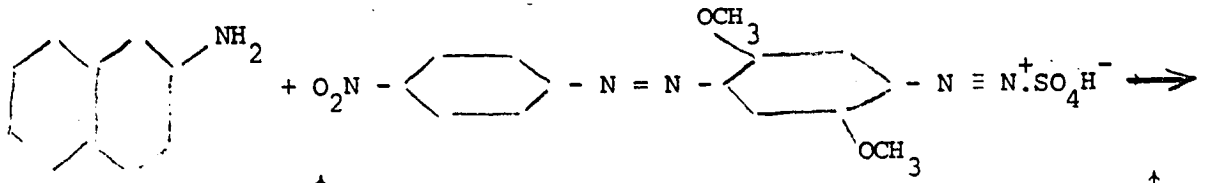
The frequencies of the active alleles of the three peptidase loci in the Parnes population were in 1976 (Loukas et al., 1978) as follows:

	Alleles	Frequencies
Lap	1.25	0
	1.18	0
	1.11	.069
	1.06	.063
	1.00	.847
	0.92	.005
	0.86	.016
	0.69	0

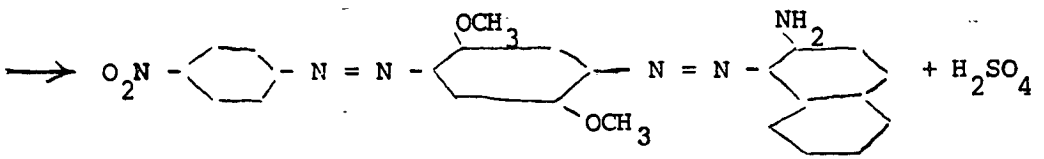
REACTION 1 (Lap)



1-leucyl-B-naphthylamide



Black K salt



REACTION 2 ($pept_1$, $pept_2$)

L-leucine-L-tyrosine $\xrightarrow{pept_1 O2\ pept_2}$ amino acids

amino acids $\xrightarrow[\text{(snake venom)}]{\text{Oxidase of amino acids}}$ $NH_3 + H_2O_2 + \ldots\ldots$

$2H_2O_2 \xrightarrow{\text{Peroxidase}} 2H_2O + O_2$

Peptidase catalyzed reactions (continued)

In vitro there are differences between peptidase 1 and 2 in the ability to cleave dipeptides as follows:

Dipeptides	Pept 1	Pept 2
L-phenylalanyl-L-leucine	++	++
L-alanine-L-aspartic acid	O	O
glycyl-L-leucine	+	++
L-methionyl-L-glutamic acid	O	++
L-leucyl-L-phenylalanine	++	++
L-alanylglycylglycine	O	O
L-alanylglycine	O	+
L-lysyl-L-aspartic acid	O	O
L-lysyl-L-phenylalanine	+	++

(with the symbol (++) is presented rapid reaction, with (+) slow reaction and with (O) no reaction).

	Alleles	Frequencies
$\underline{pept}_1$	1.60	.053
	1.00	.253
	0.40	.694
$\underline{pept}_2$	1.08	.005
	1.00	.990
	0.92	0
	0.83	.005

N (Number of tested chromosomes for every locus) = 190.

If one hypothesis is that each allele of the three loci has a different function then there are:

$$\{\binom{3}{2} + \binom{3}{1}\} \times \{\binom{4}{2} + \binom{4}{1}\} \times \{\binom{8}{2} + \binom{8}{1}\} = 2160$$

possible genotypes (factors) for modifying the M.E. locus products. This can be the maximum number of heat-sensitive phenotypes of Me after peptidase action. When highly inbred strains are used (the case in many publications) as material, this maximum number of factors decreases to 8 x 4 x 4 = 128. Within these strains the genetic material is homozygous and that can be a reason for repeatability in heat-sensitive phenotypes within the strains or in F_1 crosses. According to the earlier hypothesis I should be able to discover more hidden heat-sensitive polymorphism in the Me case by using more sensitive screening heat sensitive techniques.

An interesting aspect of the Me variation is that the peptidase concentration may vary in different parts of the body of the fly. In D. subobscura the Est 9 alloenzymes are electrophoretically detectable only in the head of the fly, while the rest of the body does not show any reaction (Loukas and Krimbas, 1975). The same is true for the two faster migrating esterase bands in D. melanogaster (personal

observations by using Poulik Buffers system).

In both these instances the differences in enzyme concentration must be at least as great as the difference in weight between the head and the rest of the body of the fly. Nothing is yet known about the heat stability of the peptidase loci products, but some of these must be active at least in crude extracts at 54°C.

It is now necessary to consider whether there is a causal relationship between the increased heat-sensitivity of Ao enzymes observed in strain K98 at 54°C over that detected at 72°C and the action of the peptidase enzymes at the lower temperature.

As reasoned above, it is possible we are increasing the polymorphism of the Me locus by a factor (S), where S is the number of the different genotypes of the Lap, $pept_1$ and $pept_2$ loci at 54°C. On how many other enzymes in crude extract experiments can these peptidase enzymes act thereby artificially increasing the level of polymorphism and therefore biasing the interpretation of the results? One possible answer is on any one and all of them.

Interactions between alloenzymes can appear even when we heat the gel after electrophoresis by the following hypothesized mechanism: If there are approximately 5,000 alloenzymes or proteins produced by a single fly and each is assumed monomorphic, when one considers that every enzyme occupies a 2mm space on the gel, even if every enzyme does not overlap another we need a gel 10 meters long for complete separation of each enzyme. Our gels usually have an average length of 5 cm, which could mean that in the same 2 mm space there may be 200 additional enzymes.

Of course the method of heating the gels is better than the crude extract technique but I think this factor must be taken into account.

According to the chromosomal homology (Table II) by using enzymatic loci from the species D. subobscura, D. melanogaster and D. pseudoobscura up to now most of the examined loci for heat-sensitive treatments (Est C, Est 6, Xdh, Pgm and Me^{Ao}) are located on the same chromosome or even the same arm, including the two main polymorphic loci Lap (Lap$_1$, Lap$_2$ in D. melanogaster) and pept$_1$ (same linkage group). So lines isogenic for these loci will be also isogenic for the two peptidase loci and therefore the interaction between the two groups will be genetically uniform. This may be an explanation for Cochrane's (1976) segregation when he described a linked factor which effects the Est-6 heat-sensitive polymorphism in D. melanogaster. In the case of my isogenic lines and other experiments done with other Drosophila species there are some very important differences.

The two peptidase loci (Lap and pept$_1$) located into the inversions O_{3+4} and O_{3+4+8} are in linkage equilibrium in the Parnes population (Loukas et al., 1978 in press) and the same must be true for the Crete population. Between the segment II (loci Xdh, Ao, Me and Odh) and the segment I (loci Lap and pept$_1$) our balanced strain has a free recombinations chromosomal segment f_2 (Figure 3) still unknown in length (C = ?). In both populations the frequency of the inversion O_{3+4} is high. The mutations which we use for isogenic lines extraction are also located in the chromosomal segment II (cu, Ba; Figure 2). As a result the segment II is isogenic in my lines, the same is not true for segment I in which the loci Lap and pept$_1$ may be homozygous or heterozygous. This can be a reason for variation in repeatability in the heat sensitive phenotypes of some strains in the Me case at least.

Xanthine dehydrogenase

Non repeatable heat-sensitive phenotypes were observed at this locus for some strains (see Table XIII)and the results of the F_1 crosses were not always homogeneous. Segregation analysis of the test cross shows no Mendelian segregation of one locus.

Octanol dehydrogenase

For this enzyme I did not succeed in producing clear readable gels because the blue stain covered the whole gel. The single fly technique gave me even worse results.

As I mentioned, the heat sensitive method is used not only for its simplicity as a technique, but also because the temperature is one of the main environmental factors influencing the ecology of Drosophila. Our sampling location in Crete is hotter throughout the year than the Parnes location. If "hidden-heat-sensitive-polymorphism" exists and is associated with the fitness of the flies I should have observed between these two genetically different populations more thermostable forms in Crete than in Parnes. However, this was not so. The results of this test are presented in Table XIV.

Another point worth mentioning here is that we do not know how many loci control the synthesis of the final enzyme product which we observe on gels. I will present here two opposite examples in which biased estimations of "hidden polymorphism" and biased conclusions will be the result if we do not know the structure and composition of the tested enzymes.

(a) In D. melanogaster except for the separate genetic control for the loci Xdh (map position 52), Ao (map position 56.6) and Po. (Pyridoxal oxidase map position 57) located on the 3R arm on the III

TABLE XIV

Homogeneity (χ^2 test) of heat sensitive phenotypes (mass technique) between the Crete and Parnes populations for the loci: (a) Ao, (b) Me and (c) Xdh.

	Crete		Parnes				
	Ao*						
Phenotypes	obs.	exp.	obs.	exp.	χ^2	d.f.	P
++-							
+--	9	5.5	4	7.5			

+++	46	49.5	71	67.5			
					4.28	1	.05-.02
	Me						
Phenotypes	obs.	exp.	obs.	exp.	χ^2	d.f.	P
---	8	5.5	5	7.5			
+--	13	13	17	17			
++-	11	14	21	18			
+++	23	23	32	32			
					3.11	3	.50-.25
	Xdh						
Phenotypes	obs.	exp.	obs.	exp.	χ^2	d.f.	P
++-							
+--	7	8	11	10			

+++	45	44	59	60			
					0.27	1	.75-.50

*In this case the χ^2 test is significant in that the results are contrary to our tested hypothesis, i.e. more heat sensitive isogenic strains than expected were observed in the population of the hotter location (Crete) and less in Parnes.

chromosome, there is common genetic control of the l.x.d. (III chromosome, map position 33) and ma-l (X chromosome map position 64.8) loci in regard to the specific activity of their enzyme (Collins et al., 1971). So polymorphism at the two latter loci (in the sense of altering the specific activity) is added to the existing polymorphism at the three former loci. If in this system we construct isogenic lines, and polymorphism exists in activity variants of the lxd locus, then linkage disequilibrium will be detected between the Xdh, A_O and P_O loci (i.e. active or less active alleles of these loci will co-occur in strains).

(b) In E. coli the RNA polymerase holoenzyme is a multisubunit enzyme with the composition $a_2bb'6$. This enzyme consists of a core polymerase (a_2bb') and a dissociable initiation factor (6). The structural loci for the b (rpoB) and b' (rpoG) subunits are located at 88.5 min on the E. coli linkage map. The structural locus for the a_2 subunit is located at 72 min.

Burdish and Berger (1975), using a thermosensitive strain, showed that this strain possesses an RNA polymerase 6 (sigma) subunit with an activity 4 - 6 times less thermostable at 45°C than the 6 from the wild type strain.

By using this strain Gross et al. (1978) mapped the 6 subunit's structural locus at 66 min. It is obvious in this case that we have three independent structural loci rather than one acting to produce the RNA polymerase enzyme.

If similar mechanisms are proved for the enzymes used in population genetic studies of Drosophila we add to our hypothetical structural locus the polymorphism of more than one other structural locus and as

a result artificially increase the polymorphism at the hypothetical structural locus, biasing interpretation of results at this locus. In addition, Beckenbach and Prakash (1977) did not discover any additional allelic variation at both of the hexokinase loci in D. pseudoobscura and D. persimilis by using (a) 135 strains from the two species from six localities and (b) heat-treatment, different starch concentrations and pCMSA factors. It is therefore probably too early to describe the 'heat-sensitive-polymorphism' as "hidden polymorphism" of any examined "structural locus". It is necessary to learn more about enzyme composition and interactions, even after a simple heat treatment of crude extracts, before interpreting this as "hidden polymorphism" and using it to support hypotheses in population genetic studies.

ADDENDUM

After this thesis was typed I was informed that G.B. Johnson gave a talk in the Aarhus Conference (workshop in Population Genetics from 14th to 17th August, 1978) about his experiments with D. melanogaster. He extracted from wild males isogenic lines for the III chromosome and observed linkage disequilibria between the Xdh and A.O. loci. More active forms at both loci were found more frequently together and the same was true for less active forms. By changing the rest of the genome he observed that this was due to modifier genes located on III and other chromosomes.

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