EVIDENCE FOR LINKAGE DISEQUILIBRIUM MAINTAINED BY SELECTION IN TWO NATURAL POPULATIONS OF *DROSOPHILA SUBOBSCURA*[1,2]

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ABSTRACT

One island and one mainland population of *Drosophila subobscura* were found polymorphic at the XDH (xanthine dehydrogenase) and the AO (aldehyde oxidase) loci. It was observed that one allele at the XDH locus, which has a low frequency in both populations, is nonrandomly associated with the alleles at the AO locus. Two lines of evidence support the thesis that this linkage disequilibrium is due to epistasis rather than random drift: (1) D or r, measures of the disequilibrium, have the same sign and magnitude in both populations. (2) The linkage disequilibrium is not due to inversions. Inversions segregating on the chromosome carrying XDH and AO have been separated into two classes, between which exchange of alleles at the two loci is suppressed. Linkage disequilibrium for XDH and AO was observed within each class. In the absence of any exchange of alleles, these disequilibria must have arisen and been maintained independently. The suggestion is made that the epistatic disequilibrium results from the close structural and physiological relationship which exists between the two enzymes.

THE introduction of electrophoretic techniques into experimental population genetics (WRIGHT 1963) opened the possibility of a direct attack on many well-developed but virtually untested theoretical concepts. The theory of "linkage disequilibrium" has been well worked out for the case of two loci (KIMURA 1956; LEWONTIN and KOJIMA 1960; BODMER and FELSENSTEIN 1967; KARLIN and FELDMAN 1970), and considerable effort has been directed toward cases of more than two loci (LEWONTIN 1967; FRANKLIN and LEWONTIN 1970). The theory predicts that non-additive interactions, i.e., epistasis, among fitness at different loci would result in excess of certain gametic types and in shortage of others, provided that the loci are closely linked.

One of the major achievements that followed the introduction of electrophoresis into population genetics was the discovery that sexually breeding populations

[1] Dedicated to George Gaylord Simpson, leading paleontologist and evolutionist, on the occasion of his seventieth birthday.

[2] This is paper No. VI of the series: The genetics of *Drosophila subobscura* populations.

contain large amounts of genetic variation (HUBBY and LEWONTIN 1966; HARRIS 1966; JOHNSON *et al.* 1966). If all these loci are maintained by overdominance, and fitness between loci is multiplicative, then, as LEWONTIN and HUBBY (1966) pointed out, an extremely high segregation load would result. To get around this problem, KIMURA (1968) and KING and JUKES (1969) hypothesized that most of the variation is not adaptive and KOJIMA and YARBROUGH (1967) hypothesized that frequency-dependent selection may be important. Segregational load could reasonably be reduced, however, if there were not independent segregation among loci, i.e., if linkage disequilibrium were built in the population. Computer models developed by FRANKLIN and LEWONTIN (1970) and WILLS, CRENSHAW and VITALE (1970) can account for the maintenance of large numbers of heterotic loci, but also lead to the conclusion that the chromosome is organized in groups of tightly linked genes.

It appears, therefore, that if heterosis and epistasis are responsible for the large amounts of variation observed, non-random associations among loci should be abundant in nature. Short-term non-random associations also are expected in populations of small size merely because of random drift (HILL and ROBERTSON 1968). However, at the present time, no experimental work has succeeded in establishing a case of linkage disequilibrium between two loci not included in or not linked to different inversions. It has been shown that strong associations exist between the alleles at three loci and the inversions segregating on the third chromosome of *Drosophila pseudoobscura* (PRAKASH and LEWONTIN 1968, 1971). The same is true for at least one locus in *Drosophila pavani* (NAIR and BRNCIC 1971) and *Drosophila subobscura* (LOUKAS and KRIMBAS, in preparation) although this is not the case for αGPDH and inversion C, which includes αGPDH, in *Drosophila melanogaster* (MUKAI, METTLER and CHIGUSA 1971). KOJIMA, GILLESPI and TOBARI (1970) examined nine locus-inversion pairs in *Drosophila melanogaster* and found that the linkage disequilibrium drops drastically as the distance between the locus and the proximal breakage point increases. MUKAI, METTLER and CHIGUSA (1971) got comparable results using the same organism. But they found non-random association between ADH and inversion C, where the map distance between the locus and the breakage point is about 30 centimorgans.

It is clear that in all the above cases we cannot assess the relative importance of epistasis and recombination suppression in maintaining those disequilibria. In one free-of-inversions case, a linkage disequilibrium between two closely linked loci, created by the experimentalists in a cage-population, decayed as soon as the gene frequencies reached equilibrium (O'BRIEN and MACINTYRE 1971). The latter case stands in contrast to the data obtained by CANNON (1963). In this experiment a few third chromosomes marked with five mutants were introduced into a wild-type population of *Drosophila melanogaster*. A significant increase of the frequency of the gametic type carrying the three middle genes was observed in all three replicas. Whatever the explanation for this increase is, the experiment shows that linkage disequilibrium can be generated in sexually breeding populations.

In this paper we report a case of a linkage disequilibrium between two loci in two natural populations of *Drosophila subobscura*. We furnish evidence which, in our view, supports the thesis that the disequilibrium is maintained by natural selection. The evidence stems from the fact that both populations exhibit the same kind of disequilibrium, as well as from the associations of the two loci with the inversions segregating at the same chromosome.

MATERIALS AND METHODS

The electrophoretic methods of detecting allozyme polymorphism at XDH and AO were the same as described by PRAKASH, LEWONTIN and HUBBY (1969) with the exception that no KCN was used for XDH and benzaldehyde was used as substrate for AO instead of acetaldehyde.

A stock of *Drosophila subobscura* homozygous for the recessive mutants, cherry (*ch*) and curly (*cu*), on the O chromosome, and also homozygous for the inversion O_{3+4}, was used for this study. We will refer to it as *chcu* stock. The stock was found to be homozygous for $XDH^{.92}$ and $AO^{.87}$. In order to determine in which chromosome loci XDH and AO are, a number of pair matings were established, using wild-caught males and *chcu* females. After larvae had appeared in the culture, the males were assayed for XDH and AO. Sons of crosses in which the male parent was homozygous for $XDH^{.94}$ and $AO^{.89}$ were backcrossed to *chcu* females. All wild-type offspring of the latter cross examined were found to be $XDH^{.94}/XDH^{.92}$ and $AO^{.89}/AO^{.87}$, while all *chcu* offspring were homozygous for $XDH^{.92}$ and $AO^{.87}$. Since recombination is suppressed in the male, we conclude that XDH and AO are in the O chromosome.

Mapping of XDH and AO was performed on the progeny of the cross:

$$♀♀ \frac{O_{3+4} ++ \; XDH^{.94} \; AO^{.89}}{O_{3+4} \; chcu \; XDH^{.92} \; AO^{.87}} \times ♂♂ \frac{O_{3+4} \; chcu \; XDH^{.92} \; AO^{.87}}{O_{3+4} \; chcu \; XDH^{.92} \; AO^{.87}}.$$

Equal numbers of (++) and (chcu), as well as (+ cu) and (ch +) were examined. Because it is known that there are differences among larval viabilities of wild and mutant phenotypes, we preferred to fix the ratio (recombinants for markers)/total in the examined flies to 0.42, which is the known map distance between *ch* and *cu*, rather than to examine numbers proportional to their viabilities. The results of the mapping are given in Figure 1. Fourteen recom-

mapping data

+	+	XDH .94	AO .89	44	+	cu	XDH .92	AO .87	14
+	+	XDH .92	AO .87	1	+	cu	XDH .94	AO .89	7
ch	cu	XDH .92	AO .87	43	+	cu	XDH .94	AO .87	12
ch	cu	XDH .94	AO .89	2	other classes				0
ch	+	XDH .92	AO .87	20			total		155
ch	+	XDH .94	AO .89	10	XDH-AO distance: 9.03 centimorgans				
ch	+	XDH .92	AO .89	2	Exact 95% conf. limits on XDH-AO distance: 5.03 – 14.69 centimorgans				

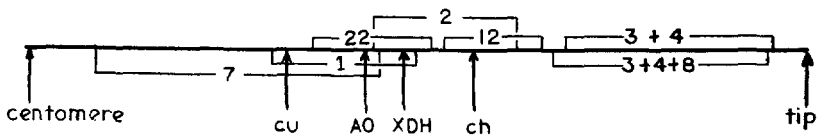

FIGURE 1.—The map of chromosome O of *Drosophila subobscura* and the mapping data of XDH and AO.

binants between XDH and AO were recovered among 155 analyzed chromosomes. DICKINSON (1970) examined 256 chromosomes and obtained twelve recombinants between the homologous loci in *D. melanogaster*. The two estimates of map distance, i.e., 9.0 in *D. subobscura* and 4.7 in *D. melanogaster*, are not different statistically. The relative positions of the inversions shown in Figure 1 were obtained from the map published by KUNZE-MÜHL and MÜLLER (1958) while the positions of the markers were inferred from the data of KOSKE and MAYNARD SMITH (1954).

Natural populations were sampled directly by trapping adults on banana bait. Every wild-caught male was crossed to *chcu* females. A single male progeny from each cross was backcrossed to *chcu* females. Salivary-gland chromosomes were examined from the larvae developed from the backcrosses. One to eight larvae were examined from each cross until the first heterozygote for inversions at O chromosome was encountered. If all eight individuals were homozygous for inversions, the wild chromosome was assigned as being O_{3+4}. This procedure produces a negligible ($\sim$.004) overestimation of the frequency O_{3+4}. Wild-type progeny from each backcross were assayed for XDH and AO.

Two natural populations were sampled, the first from the village of Alikianou on the island of Crete (Greece) and the second from the mountain of Parnes about 15 miles north of Athens (Greece). The two populations are separated by about 220 miles, half of which is open sea. Both populations have been studied in the past for changes in inversion frequencies (KRIMBAS 1964, 1967; KRIMBAS and ALEVIZOS 1972). Empirical evidence, mainly high yield of trapping, suggests that the Parnes population is a flourishing one. For the population of Crete, preliminary data by DIAMANTOPOULOU and KRIMBAS on the allelism of lethals suggest that this population is also large.

RESULTS

Every wild chromosome was examined for inversions and the allele it carried at the XDH and AO locus. Alleles at XDH and AO were given the names .92, .87, etc., according to their mobility relative to a standard ($XDH^{1.00}$ and $AO^{1.00}$ of *Drosophila pseudoobscura*, PRAKASH, LEWONTIN and HUBBY 1969). O_{ST}, O_{3+4}, O_{3+4+8}, etc., stand for different gene arrangements of the O chromosome. A knowledge of the inversions of this chromosome and the linkage relationships to each other as well as to XDH and AO is necessary in order to understand the analysis of the data. This chromosome is homologous to the second chromosome of *Drosophila pseudoobscura* and to 3R of *Drosophila melanogaster* (ZOUROS *et al.*, in preparation). The various inversions of the chromosome are shown in Figure 1. Even though (3 + 4) does not overlap with (1), (2), (7), or (22), it is very uncommon to find one of the latter four inversions without (3 + 4). O_{3+4+7} then stands for a gene arrangement containing inversions (3 + 4) and (7). Inversion (8) overlaps (3 + 4) so that (3 + 4 + 8) must be regarded as one inversion. This non-random association between non-overlapping inversions in *D. subobscura* will be examined in detail in a separate paper. For the purposes of this work the gene arrangements of the O chromosome can be separated into two classes:

I. O_{ST}, O_{3+4}, O_{3+4+8}
II. O_{3+4+2}, O_{3+4+22}, O_{3+4+1}, O_{3+4+7}.

In a female heterozygous for gene arrangements of the first class there is no crossover suppression in the XDH–AO region. Heterozygotes for an arrangement of the first class and an arrangement of the second have crossing over in this region completely suppressed. It is quite possible that complete crossover suppres-

TABLE 1

*Gametic types in Crete population**

Gene arrangement	AO	XDH .88 (.069)	.89 (.015)	.90 (.031)	.92 (.809)	.94 (.061)	.96 (.015)	Totals
O_{3+4+8} (.817)	.82	.	.	.	1	.	.	1
	.83	.	.	1	2	.	.	3
	.85	.	.	.	1	.	.	1
	.87	7	1	1	69	1	1	80
	.89	1	.	1	10	5	.	17
	.91	.	1	.	3	.	1	5
		8	2	3	86	6	2	107
O_{3+4} (.099)	.85	.	.	.	.	1	.	1
	.87	.	.	.	10	.	.	10
	.89	.	.	.	.	1	.	1
	.91	.	.	.	1	.	.	1
		.	.	.	11	2	.	13
O_{ST} (.038)	.87	.	.	1	2	.	.	3
	.89	.	.	.	1	.	.	1
	.91	.	.	.	1	.	.	1
		.	.	1	4	.	.	5
O_{3+4+1} (.038)	.85	.	.	.	1	.	.	1
	.87	1	.	.	3	.	.	4
		1	.	.	4	.	.	5
O_{3+4+22} (.008)	.87	.	.	.	1	.	.	1
Totals		9	2	4	106	8	2	131

	AO					
Allele	.82	.83	.85	.87	.89	.91
Number in the sample	1	3	3	98	19	7
Frequency	(.008)	(.023)	(.023)	(.748)	(.145)	(.053)

* In parentheses are the allelic or gene-arrangement frequencies.

sion at the XDH–AO region exists in heterozygotes for arrangements of the second class.

Crete population: Table 1 contains the data of the Crete population. The number of any gametic type can be obtained from this table. Frequencies of gene arrangements and alleles are given in parentheses. In testing these data for linkage disequilibrium, we are faced with the problem of the rare alleles. One approach could be to discard gametic types with frequency lower than 0.05. This is equivalent to ignoring more than 25% of the sample. Rather we preferred the following approach. In constructing the tables on which the χ^2 test of goodness-of-fit is performed, we pooled together alleles starting with those of the lowest frequency and proceeding until the "expectation" of any cell was higher than 5. For 2 × N tables this requirement was dropped as not being necessary (LEWONTIN and FELSENSTEIN 1965). A χ^2 test performed on pooled classes in most cases gives less significant results than if performed on single classes. This means that

TABLE 2

χ^2 tests on the data of the Crete population

XDH	AO .87	AO .89	Others
.92	85 (79.30)	11 (15.37)	10 (11.33)
Others	13 (18.70)	8 (3.63)	4 (2.67)
	$\chi^2_2 = 9.469$	$.01 > P > .005$	

a

	XDH .88	XDH .92	XDH .94	Others
O_{3+4+8}	8 (7.35)	86 (86.58)	6 (6.53)	7 (6.53)
Others	1 (1.65)	20 (19.42)	2 (1.47)	1 (1.47)
	$\chi^2_3 = 0.752$	$.8 > P > .9$		

b

XDH	AO .87	AO .89	Others
O_{3+4+8}	80 (80.04)	17 (15.52)	10 (11.44)
Others	18 (17.96)	2 (3.48)	4 (2.56)
	$\chi^2_2 = 1.761$	$.5 > P > .4$	

c

XDH	Excluding XDH$^{.94}$ AO .87	AO .89	Others
.92	85 (83.60)	11 (11.20)	10 (11.20)
Others	12 (13.40)	2 (1.80)	3 (1.80)
	$\chi^2_2 = 1.124$	$.3 > P > .2$	

d

if a test on pooled classes is significant, we can be almost sure that it would be equally or more significant if it were performed on single classes.

The χ^2 tests are shown in Table 2(a,b,c). Chromosomal arrangements are randomly associated with alleles at XDH and AO locus. On the other hand, there is significant non-random association between alleles at the two loci. Over 80% of the chromosomes in the Crete population are O_{3+4+8}. Even if we disregard the other 20% of the chromosomes, the level of significance of the non-random association between the alleles at these two loci does not change. Therefore, the linkage disequilibrium in this population is not due to inversions. Having established this we may ask if all alleles at the two loci contribute equally to the linkage disequilibrium. An inspection of Table 1 reveals that XDH$^{.94}$ is the allele with the most "aberrant" behavior. This allele was found eight times in the sample. In six cases it was with AO$^{.89}$, an allele of AO with frequency 0.145, and only once with AO$^{.87}$, the most frequent allele of AO. If we discard from the sample the gametes, which carry XDH$^{.94}$, the disequilibrium disappears. This test is given in Table 2d. This result cannot be obtained by discarding any other single allele.

Parnes populations: Table 3 gives the data for the population from Parnes. The χ^2 tests are given in Table 4. Any pair-wise comparison reveals significant linkage disequilibrium. There is non-random association not only between XDH and AO, but also between XDH and arrangements, and AO and arrangements. We may then ask if the disequilibrium between XDH and AO is due to inversions. Examination of the pair XDH-arrangements reveals that we cannot find an allele of XDH which, when excluded from the sample, will eliminate the disequilibrium. The same is true for the pair AO-arrangements. On the contrary, if we take out of the sample the gametes which carry the gene arrangement O_{3+4+1} both tests, i.e., XDH-arrangements and AO-arrangements, give a remarkably low χ^2 (Table 4d,e). Thus the disequilibria between arrangements and loci can be totally attributed to O_{3+4+1}. This is not a mere coincidence. From Figure 1 we see that inversion (1) includes both XDH and AO. This means that O_{3+4+1} does not exchange alleles at XDH and AO with either members of class I or members of class II. The result is that O_{3+4+1} is rather poor in allelic variation, containing only two alleles at each locus, while O_{ST}, although it is less frequent than O_{3+4+1}, contains five alleles at each locus. The low frequency of the O_{3+4+1} arrangement in the Crete population made any similar disequilibria between loci and inversions difficult to detect.

Having established that O_{3+4+1} can totally account for the arrangement-locus disequilibria, we may ask whether or not the exclusion of O_{3+4+1} from the sample could also eliminate the XDH-AO disequilibrium. The test is given in Table 4f. The XDH-AO disequilibrium remains significant after excluding O_{3+4+1}. The validity of the test is questionable, however, since two expectations are less than five in this 3×3 table. But we may observe that XDH$^{.94}$ shows a strong tendency to combine with AO$^{.89}$. This tendency is present in the whole sample from Parnes and persists in the sample after removing O_{3+4+1}. This part of the sample contains ten XDH$^{.94}$-AO$^{.89}$ gametes. Given that under random association this

TABLE 3

*Gametic types in Parnes population**

Gene arrangement	AO	XDH .86 (.013)	.88 (.146)	.90 (.099)	.92 (.623)	.94 (.093)	.96 (.026)	Totals
O_{3+4+8} (.060)	.85	.	2	.	.	.	.	2
	.87	.	.	1	5	.	.	6
	.89	.	.	.	.	1	.	1
		.	2	1	5	1	.	9
O_{3+4} (.424)	.83	.	.	.	1	.	.	1
	.85	.	1	1	4	.	.	6
	.87	2	7	5	18	1	1	34
	.89	.	.	3	8	5	1	17
	.91	.	1	1	4	.	.	6
		2	9	10	35	6	2	64
O_{ST} (.159)	.83	.	.	1	.	.	.	1
	.85	.	.	.	3	1	.	4
	.87	.	2	2	9	1	2	16
	.89	.	1	.	.	1	.	2
	.91	.	.	.	1	.	.	1
		.	3	3	13	3	2	24
O_{2+4+1} (.238)	.87	.	6	.	29	.	.	35
	.89	.	1	.	.	.	.	1
		.	7	.	29	.	.	36
O_{3+4+22} (.079)	.85	.	.	1	.	.	.	1
	.87	.	.	.	6	1	.	7
	.89	.	.	.	3	1	.	4
		.	.	1	9	2	.	12
O_{3+4+7} (.033)	.87	.	1	.	2	.	.	3
	.89	.	.	.	.	2	.	2
		.	1	.	2	2	.	5
O_{3+4+2} (.007)	.87	.	.	.	1	.	.	1
Totals		2	22	15	94	14	4	151

	AO				
Allele	.83	.85	.87	.89	.91
Number in the sample	2	13	102	27	7
Frequency	(.013)	(.086)	(.675)	(.179)	(.046)

* In parentheses are the allelic or gene-arrangement frequencies.

gamete should have a frequency of 0.028, the probability of getting ten or more in a sample of 115 gametes is 0.0015. Furthermore, if we exclude from the whole sample gametes carrying XDH$^{.94}$, the XDH-AO disequilibrium disappears (Table 4g). These observations suggest that the XDH-AO linkage disequilibrium cannot be simply attributed to O_{3+4+1}, or to inversions generally. Rather it is due to the tendency of XDH$^{.94}$ and AO$^{.89}$ to combine preferentially. This is the same tendency responsible for the linkage disequilibrium in the population of Crete.

TABLE 4

χ^2 tests on the data of the Parnes population

XDH	AO .87	AO .89	Others
.92	70 (63.50)	11 (16.81)	13 (13.69)
Others	32 (38.50)	16 (10.19)	9 (8.30)

$\chi^2_2 = 7.177$ $.05 > P > .025$

	XDH .88	XDH .92	Others
O_{3+4}	9 (9.32)	35 (39.84)	20 (14.84)
O_{3+4+1}	7 (5.25)	29 (22.41)	0 (8.34)
Others	6 (7.43)	30 (31.75)	15 (11.82)

$\chi^2_4 = 14.511$ $.01 > P > .005$

	AO .87	AO .89	Others
O_{3+4}	34 (43.44)	17 (11.44)	13 (9.32)
O_{3+4+1}	35 (24.32)	7 (6.44)	0 (5.25)
Others	33 (34.45)	9 (9.12)	9 (7.43)

$\chi^2_4 = 21.055$ $P < .001$

XDH	Excluding O_{3+4+1} O_{ST}	Others
.92	35 (36.17)	13 (13.57)	17 (15.26)
Others	29 (27.83)	11 (10.43)	10 (11.74)

$\chi^2_2 = .598$ $.8 > P > .7$

XDH	Excluding O_{3+4+1} AO .87	AO .89	Others
O_{2+4}	34 (37.29)	17 (14.47)	13 (12.24)
Others	33 (29.71)	9 (11.53)	9 (9.76)

$\chi^2_2 = 1.757$ $.5 > P > .4$

AO	Excluding O_{3+4+1} XDH .88	XDH .92	Others
.87	10 (8.74)	41 (37.87)	16 (20.39)
.89	1 (3.39)	11 (14.70)	14 (7.91)
Others	4 (2.87)	13 (12.43)	5 (6.70)

$\chi^2_4 = 9.593$ $.05 > P > .025$

XDH	Excluding XDH·92 AO .87	AO .89	Others
.92	70 (67.93)	11 (11.66)	13 (14.41)
Others	29 (31.07)	6 (5.34)	8 (6.59)

$\chi^2_2 = .760$ $.7 > P > .6$

TABLE 5

χ^2 tests on the to classes of the Parnes population

[a] XDH

Class	.88	.90	.92	Others
I	14 (14.13)	14 (9.64)	53 (60.38)	16 (12.85)
II	8 (7.87)	1 (5.36)	41 (33.62)	4 (7.15)

$\chi^2_3 = 10.203$ $.01 > P > .005$

[b] AO

Class	.87	.89	Others
I	56 (65.52)	20 (17.34)	21 (14.13)
II	46 (36.48)	7 (9.66)	1 (7.87)

$\chi^2_2 = 14.344$ $P < .001$

[c] Class II XDH

	.92	Others
O_{3+4+1}	29	7
Others	12	6

$P \simeq .215$*

[d] Class II AO

	.87	Others
O_{3+4+1}	35	1
Others	11	7

$P \simeq .001$*

[e] Class I XDH

AO	.88	.92	Others
.87	9 (8.08)	32 (30.60)	15 (17.32)
.89	1 (2.89)	8 (10.93)	11 (6.19)
Others	4 (3.03)	13 (11.47)	4 (6.49)

$\chi^2_4 = 8.363$ $.10 > P > .05$

[f] Class II XDH

AO	.92	Others
.87	38	8
Others	3	5

$P \simeq .021$*

* By Fisher's exact test.

We mentioned earlier that the gene arrangements of the O chromosome can be separated into two classes according to the presence or absence of crossover suppression in the XDH-AO region. We may ask whether or not the two classes are homogeneous with respect to allelic frequencies of XDH and AO. The tests are given in Table 5(a,b). The two classes are heterogeneous for both loci. It is then clear that the presence of inversions suppressing crossing over in the XDH-AO region separated the population into two heterogeneous classes in respect to these loci. The test of homogeneity can be extended within the classes. Alleles at XDH and AO are distributed among the three types of class I, as should be expected under the hypothesis of randomness, in agreement with the hypothesis that in the absence of crossover suppression there would be no differentiation in the allelic frequencies. The inversions of class II are different in the sense that some of them include one or both loci. O_{3+4+1} tested against the rest of the arrangements of class II is found to be different for AO but not for XDH (Table 5c,d). Since class I and class II are heterogeneous for the allelic frequencies we may ask whether or not this difference has any effect on the linkage disequilibrium between XDH and AO. The disequilibrium is significant in class II, but it only approaches significance in class I (Table 5e,f). The pair $XDH^{.94}$-$AO^{.89}$ is still in excess in class I. The probability of getting seven or more gametes of this type in class I is 0.0047. The frequency of this gamete in classes I and II is 0.072 and 0.056, respectively. These frequencies are not different statistically. We may infer that a larger sample could give a test for linkage disequilibrium in class I as significant as it is for the whole population.

Finally, we can compare the two populations. The differences in the gene arrangements are so obvious that they need not be tested. The heterogeneity of the populations is also significant for XDH and approaches significance for AO (Table 6a,b). These differences cannot be attributed to differences in inversions since they do not disappear if we compare class I of Parnes and Crete. The differences indicate that if there is any migration between the two populations it is not enough to make the populations homogeneous in regard to allelic frequencies at XDH and AO. In contrast to these differences, Crete, class I of Parnes, and class II of Parnes are not different in respect to the number of $XDH^{.94}$-$AO^{.89}$ chromosomes they contain (Table 6c).

DISCUSSION

Linkage disequilibrium among alleles at two loci in a population can be the result of epistasis or random drift. In most cases it is difficult to distinguish between these two factors. To get around this problem two different approaches have been used: following the same population for a number of consecutive generations or examining simultaneously more than one population. Both approaches have their weak points. Small differences in the magnitude of the linkage disequilibrium, observed in the same population at different times, cannot always be interpreted as indicating persistence of the disequilibrium or a gradual decay of it. Big differences, which ordinarily might be taken as indicating ran-

TABLE 6

χ^2 tests on Crete and Parnes populations

	[a] XDH				
	.88	.90	.92	.94	Others
Crete	9 (14.40)	4 (8.83)	106 (92.91)	8 (10.22)	4 (4.65)
Parnes	22 (16.60)	15 (10.17)	94 (107.09)	14 (11.78)	6 (5.35)
	$\chi^2_4 = 13.22$ $.025 > P > .01$				

	[b] AO			
	.85	.87	.89	Others
Crete	3 (7.43)	98 (92.91)	19 (21.37)	11 (9.29)
Parnes	13 (8.57)	102 (107.09)	27 (24.63)	9 (10.71)
	$\chi^2_3 = 6.52$ $.10 > P > .05$			

	[c] Gametes	
	$XDH^{.94}$–$AO^{.89}$	Others
Crete	6 (7.43)	125 (123.57)
Parnes I	7 (5.50)	90 (91.50)
Parnes II	3 (3.07)	51 (50.93)
	$\chi^2_2 = .728$ $.7 > P > .6$	

dom drift, may be the result of changes in the selection coefficients. In examining more than one population, one may find linkage disequilibrium in one population but not in the other, or may find disequilibrium in both populations but of different sign and magnitude. In neither case can random drift be ruled out. But if the same disequilibrium were found in more than one population the hypothesis of selection would be favored. Migration could be invoked to explain this similarity, but by allowing migration we assume in effect that we are not dealing any longer with separate populations. Rather we have one population with effective size such that random drift cannot generate any significant linkage disequilibrium. The populations cannot be both small enough so that non-random associations can be generated by random drift and yet exchange migrants to an extent that will prevent differentiation in the nature of the linkage disequilibrium.

It is for this reason that we have followed the second approach. Two populations were studied simultaneously. The Parnes population is a mainland population. It undoubtedly exchanges migrants with other surrounding populations. The Crete population is an island population. It may exchange migrants with other populations on the island, but the rate of gene exchange with populations outside the island is questionable. The two populations are very different for inversions segregating for all five chromosomes. These differences are persistent through time (KRIMBAS 1964, 1967; KRIMBAS and ALEVIZOS 1972). Tables 6a and 6b show that the populations are different for XDH and AO as well, and we have data extending this dissimilarity to other allozyme systems. If some migration exists, it should be of such an order that it cannot balance the selective forces

in establishing different patterns of variation. In contrast to these differences the linkage disequilibrium in the two populations is the same in almost every respect. In both cases the pair $XDH^{.94}$-$AO^{.89}$ is in excess. If we calculate a D and r, by taking $XDH^{.94}$-$AO^{.89}$ and XDH^{others}-AO^{others} as the two "coupling phases," we get for Crete, $D = 0.0371$, $r = 0.442$, and for Parnes, $D = 0.0496$, $r = 0.447$. The probability that the two correlation coefficients are the same is 0.96 and the probability that they are different from zero is 0.001. The linkage disequilibrium in the two populations is almost identical. Neither random drift within populations nor migration between the populations can easily explain this observation. If migration is to account for the similarity, it must have been so strong that it would have eliminated the differences in the inversions and the gene frequencies, which on the contrary are profound. Under such migration pressure the whole species of *D. subobscura* would approximate one huge population where linkage disequilibria could not be generated by random events. We are then left with selection as the most probable cause of the observed linkage disequilibria.

Another independent piece of evidence for selection comes from the Parnes population alone. This population can be thought of as consisting of two classes, according to the gene arrangements they contain. The two classes do not exchange alleles at XDH and AO, yet gametes of the type $XDH^{.94}$-$AO^{.89}$ were found in excess in both classes. Class II contains inversions among which there is no free exchange of alleles at XDH and AO, yet $XDH^{.94}$-$AO^{.89}$ gametes were found in two out of the three gene arrangements of class II (a fourth arrangement, O_{3+4+2}, was found only once in the sample). Double crossing over cannot explain the presence of this combination in almost all types of inversions. Nor is its frequency low enough to be explained by mutation alone. The excess of $XDH^{.94}$-$AO^{.89}$ in both classes must have arisen and been built up independently. If random drift is to account for the linkage disequilibrium in this population, it had to be of the same kind in both classes, an event quite improbable for a random phenomenon. Selection provides a far more convincing explanation. The two classes of the Parnes population can be thought of as being two different "gene pools" in regard to loci in the XDH-AO region. But they are identical in every other respect. They freely exchange alleles in the rest of the genome and they live under the same environment. Therefore they are subject to the same forces of selection which have established the same linkage disequilibrium.

According to the two-locus theory, epistatic linkage disequilibrium can be expected only between closely linked loci. We have estimated the distance between XDH and AO at 9 centimorgans. The effective distance, therefore, is 4.5 centimorgans, since there is no crossing over in the males of Drosophila. On the other hand, if the epistasis is strong, it can generate linkage disequilibrium between loci far apart on the chromosome (LEWONTIN 1964). An important concomitant of the observation that the linkage between XDH and AO is maintained by epistasis is that the genic variation observed in Mendelian populations is not isoallelic variation neutral to selective forces. This follows from the fact that epistasis alone cannot maintain the linkage disequilibrium unless at least one of the loci is heterotic.

One of our populations, Crete, can be regarded as being free of inversions in respect to XDH-AO region, since only 4.5% of the chromosomes carry inversions affecting this region. This percentage is 35.8 in the population of Parnes. The separation of this population into two classes helped us to show that the linkage disequilibrium between XDH and AO is not due to inversions. But it also demonstrated the importance of the inversions in determining different patterns of variations at the loci they include. Classes I and II are different in their allelic frequencies at XDH and AO. In this respect our results are in agreement with the well established fact that there is direct association between inversions and allelic variation.

The linkage disequilibrium that we have observed in two populations can be attributed totally to the tendency of $XDH^{\cdot 94}$ to combine with $AO^{\cdot 89}$. Allele $XDH^{\cdot 94}$ has a frequency of 0.06 in the Crete population and of 0.09 in the Parnes population. The respective frequencies of $AO^{\cdot 89}$ are 0.14 and 0.18. Judging from their appearance on the zymogram, these alleles show no difference from the other alleles in the density of the band and the formation of the hybrid zone when in the heterozygous condition. However, xanthine dehydrogenase and aldehyde oxidase are structurally and physiologically very closely related enzymes. Together with pyridoxal oxidase they represent the products of a system of genes extensively studied in *D. melanogaster*. Mutations at three loci—rosy (*ry*), maroon-like (*ma-l*) and low (*lxd*)—affect XDH activity. Electrophoretic variants of XDH map at *ry*. COURTRIGHT (1967) and DICKINSON (1970) demonstrated that AO is a distinct enzyme and described its structural gene (Aldox), which is analogous to *ry* in almost all respects. Mutations at *ma-l* and *lxd* also affect AO activity. Although it is still unknown how the products of the three loci interact in the formation of the molecule of each enzyme, it is almost sure that the two enzymes share a common co-factor or subunit (GLASSMAN *et al.* 1968). XDH is involved in the degradation of purines to uric acid, as well as in the metabolism of pteridines (MITCHELL, GLASSMAN and HADORN 1959). To our knowledge, no information yet exists as to the role of AO in the physiology of the fly. GLASSMAN (1965) notes that XDH can catalyze the oxidation of benzaldehyde to benzoic acid. This is exactly the reaction involved in our procedure of developing the zymogram for AO. We may therefore suggest that the epistatic interaction between the two enzymes, which keeps their structural loci in linkage disequilibrium, is a result of their close relationships.

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