THE EFFECT OF COMBINING ALLELES INTO ELECTROPHORETIC CLASSES ON DETECTING LINKAGE DISEQUILIBRIUM

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ABSTRACT

When alleles are combined into few detectable classes, linkage correlations are underestimated most of the time. The probability that the linkage correlation will be underestimated is a function of the actual degree of correlation and the evenness of the allelic distribution, but is mainly determined by the distribution of alleles into distinguishable classes. With only two alleles per class this probability will usually be higher than 0.7. Also, the consistency in the sign of the linkage disequilibrium over many populations may escape detection. An increase of sample size by one order of magnitude or more may be required to compensate for the loss in detection power. It follows that the available electrophoretic studies of linkage correlations, although negative in their majority, do not suggest that epistatic interactions and linkage disequilibria are rare in natural populations.

THE possibility that associations among nonallelic genes depart from those expected by chance alone has received a great amount of attention by evolutionary biologists. Recently, WALLACE (1975) has defined it as one of the most fundamental problems of population genetics. Yet, with the exception of CANNON (1963), all studies of linkage disequilibrium among two or more loci in natural or laboratory populations have utilized electrophoretically detectable genetic variation. But electrophoresis detects only a part, possibly a small part, of protein variation, a fact for which BERNSTEIN, THROCKMORTON and HUBBY (1973), SINGH, HUBBY and THROCKMORTON (1975) and JOHNSON (1976) have provided direct evidence. Unlike the case of estimating amounts of genetic variation, it is not immediately clear what the effect of this limitation of electrophoresis is on ascertaining the degree of departure from randomness of associations among nonallelic genes.

In this paper we attempt to evaluate the significance of this effect by analyzing computer-generated data. Our results have an immediate bearing on the interpretation of the results of most studies on linkage disequilibrium that have been published and of ones to appear in the near future.

A similar problem arises when electromorphs of low frequency are pooled together by the experimenter for the construction of the χ^2 contingency test of the hypothesis of random association between electromorphs. This is a usual practice in studies of linkage disequilibrium (ZOUROS and KRIMBAS 1973; CHARLESWORTH

and CHARLESWORTH 1973; MUKAI, WATANABE and YAMAGUCHI 1974; LANGLEY, TOBARI and KOJIMA 1974). LANGLEY, TOBARI and KOJIMA (1974) commented on the problem but did not examine it at any length.

METHODS

Introductory remarks: This study assumes that gametes are being scored. HILL (1974) has shown that when diploids are being scored the information on linkage disequilibrium obtained from the complete identification of N dipliod individuals is equivalent to the information obtained from the identification of N haploid genomes. It has not yet been investigated whether or not the limitations of electrophoresis affect the efficiency of the two methods to the same extent.

Let A_i be an allele of locus A (i=1 to m), B_j an allele of locus B (j=1 to n), and p_{ij} the relative sample frequency of gamete A_iB_j. Then $p_{i.} = \sum_j p_{ij}$ and $p_{.j} = \sum_i p_{ij}$ give the sample frequency of alleles A_i and B_j respectively. The statistic $\chi^2 = N\sum_i\sum_j \frac{(p_{ij}-p_{i.}p_{.j})^2}{p_{i.}p_{.j}} = N\gamma^2$ with $(m-1)(n-1)$ degrees of freedom gives a measure of the nonrandom association in the sample. In this formulation N stands for the sample size and $\gamma^2 = \frac{\chi^2}{N}$ can be calculated from the sample estimates of gametic frequencies. In the two-allele case $\frac{\gamma^2}{(m-1)(n-1)} = r = \frac{D}{(p_{1.}p_{2.}p_{.1}p_{.2})^{1/2}}$, where r and $D = p_{11}p_{22} - p_{12}p_{21}$ are conventional measures of linkage disequilibrium.

Let the m alleles of locus A fall into k electromorphs and the n alleles of locus B into l electromorphs ($k \leq m, l \leq n$) and let q_{ij} stand for the sample frequency of the electromorph combination. A measure of the electrophoretically detectable association is given by $\chi^2 = N\sum_i\sum_j \frac{(q_{ij}-q_{i.}q_{.j})^2}{q_{i.}q_{.j}} = Nc^2$ with $(k-1)(l-1)$ degrees of freedom. If f is the number of ways m alleles can be lumped into k electromorphs and g is the number of ways n alleles can be lumped into l electromorphs then there are $f \times g$ ways of reducing the $m \times n$ matrix of allelic combinations to $k \times l$ matrices of electromorph combinations. Each of these reduced matrices gives a different level of significance for the association. This suggests that a way of looking into the effect of pooling is to observe what fraction of the reduced matrices gives a level of association lower than the original $m \times n$ matrix. If, for a fixed N, most reduced matrices give a lower association than the original matrix then the net effect of pooling would be to allow many nonrandom associations to go undetected. In these cases an increase in sample size will be required to offset the limitations of electrophoresis. But if a significant proportion of the reduced matrices result in an association of higher degree, then there is the danger of electrophoretic data generating spurious linkage disequilibria.

Noting that the χ^2 distribution with n degrees of freedom has mean n and variance $2n$ we can have the approximations

$$u_1 \simeq \frac{N\gamma^2 - n_1}{(2n_1)^{1/2}} \quad \text{and} \quad u_2 \simeq \frac{Nc^2 - n_2}{(2n_2)^{1/2}}$$

where $n_1 = (m-1)(n-1)$, $n_2 = (k-1)(l-1)$, and u_1 and u_2 are standard normal deviates of statistical significance equivalent to χ^2 obtained from the unreduced and the reduced matrix respectively. The requirement that a given combination of alleles results in loss of significance is $u_1 > u_2$, or

$$\gamma^2(n_2)^{1/2} - c^2(n_1)^{1/2} > 1/N\ (n_1(n_2)^{1/2} - n_2(n_1)^{1/2})$$

It is interesting to know how the sample size affects the frequency of those matrices which underestimate the amount of correlation. It is easy to show algebraically that for fixed N the

value of the χ^2 statistic calculated from the unreduced matrix is always larger than that calculated from any reduced matrix:

$$\chi^2_{n_1} - \chi^2_{n_2} = N(\gamma^2 - c^2) \geq 0$$

If α_{n_1} and α_{n_2} are the critical values of the two χ^2 distributions for a specified level of significance, then the requirement that the reduced matrix gives a χ^2 of lower significance is:

$$N(\gamma^2 - c^2) > \alpha_{n_1} - \alpha_{n_2}$$

Obviously, there will be a value of N beyond which any pooling will reduce the significance of the test. But as N decreases, the probability that a given combination of alleles will generate a χ^2 with a level of significance higher than the one obtained from the original matrix increases.

The approach: It becomes clear that the problem of ascertaining the effect of pooling on detecting correlations is reduced to obtaining an expression that will give for a fixed N the percentage, R_α, among all possible combinations of alleles that generate a statistical level of significance lower than α, the one obtained from the unreduced matrix. Alternatively, we may search for an expression that will give the sample size, N_p, required so that the correlation is detected (at a given level of significance) in a specified fraction, i, of possible combinations. Then, the ratio N_p/N (where N is the sample size required for the detection of the association at the same level of significance from the unreduced matrix) gives the factor by which the sample size must be increased for the detection of the association, as the result of the pooling.

In all likelihood these expressions are difficult to obtain analytically. In our computer simulations we examine the effect of three variables on the detection of linkage disequilibrium. These variables are the degree of electrophoretic resolution, the allelic distribution of the loci involved, and the actual amount of linkage disequilibrium among alleles at these loci.

For the degree of electrophoretic resolution, S, we used the expression $S = (1/m)(\sum_i (r_i/m)^2)^{-1}$, where r_i is the number of alleles falling into the ith electromorph ($\sum_i^k r_i = m$). The index of electrophoretic resolution so defined ranges from $1/m$ (all alleles having the same electrophoretic mobility) to 1 (each allele giving a separate electrophoretic band).

All simulations refer to the two-locus case. The element p_{ij} ($i=1$ to $m-1$, $j=1$ to $n-1$) of the $m \times n$ matrix of the gametic frequencies was obtained as $p_i p_j x_{ij}$, where x_{ij} was drawn from the uniform distribution:

$$p(x) = \frac{1}{2a}, \quad 1-a < x < 1+a$$
$$p(x) = 0 \quad \text{, otherwise}$$

and a was chosen in such a way so that γ^2 assumes values within a specified range. The $m \times n$ matrix was reduced to a series of "electromorph-combination" matrices according to a number of specified types of pooling. These types of pooling determine how many alleles will form one electromorph, but they do not determine which particular alleles will form that electromorph. For example, suppose that $m = n = 6$ an dthat one of the types of pooling is $A(3,3)B(5,1)$ meaning that at locus A three alleles will form one electromorph and the remaining three another one, and that in locus B five alleles will form one electromorph. The electrophoretic resolution at locus A in this example is $S_A = \frac{2}{6}$, and that at locus B is $S_B = \frac{1.385}{6}$. If we define the index of electrophoretic resolution for the matrix as $S_{AB} = S_A.S_B$, then in this example $S_{AB} = \frac{2.77}{36}$. There are ten different ways in which alleles of locus A can be pooled into two groups of three, and six ways in which alleles of locus B can form groups of five and one. This type of pooling, therefore, generates 60 reduced matrices.

The quantities $c^2{}_i$ (one for each reduced matrix) were calculated and ranked in increasing order. Let R be the ranking position, expressed as a percentage, of a given c^2, and $\alpha'_{0.05}$ and $\alpha_{0.05}$ be the 5% critical values of the χ^2 distributions with degrees of freedom equal to that of the reduced and the original matrix. If N is the sample size required for the association in the unreduced matrix to be significant at exactly the 5% level and if c^2 is such that the association

in the reduced matrix is again significant at exactly the 5% level, then $N\gamma^2 = \alpha_{0.05}$ and $Nc^2 = \alpha'_{0.05}$ from which it follows that $c^2 = \gamma^2 \frac{\alpha'_{0.05}}{\alpha_{0.05}}$. Then the value of R satisfying the relationship $c^2_R < \gamma^2 \frac{\alpha'_{0.05}}{\alpha_{0.05}} < c^2_{R+1}$ gives the percentage of reduced matrices in which the association cannot be detected at the 5% level. If we want to increase the sample size from N to N_p so that 50% of the reduced matrices would underestimate and 50% overestimate the linkage disequilibrium, then we require that $N_p c^2_{50} = \alpha'_{0.05}$ from which we obtain $\frac{N_p}{N} = \frac{\gamma^2}{c^2_{50}} \frac{\alpha'_{0.05}}{\alpha_{0.05}}$. After R and N_p/N were obtained, the original matrix was reduced again according to a new specified type of pooling. The results were expressed in terms of $\overline{R}$ and $\log_{10}(\overline{N/N_p})$, the means of all runs in which the type of pooling, the allelic distributions and the range of γ^2 were the same.

The types of pooling were chosen in such a way as to generate indices of resolution similar to or higher than the ones expected from electrophoresis (see DISCUSSION). Likewise, allelic frequency distributions were selected as to represent distributions expected under the most general models of selection, mutation and random drift. Finally, the range of γ^2 was decided on the principle that only a small fraction of the expected absolute numbers in the unreduced matrix are allowed to be smaller than one. Our analysis involves a sample, N, such that the association is detected from the unreduced matrix at the 5% level of significance, *i.e.*, $N\gamma^2 = \alpha_{0.05}$. From this we see that the requirement $Np_{l.}p_{.l} > 1$, where $p_{l.}$ and $p_{.l}$ are the lowest values in the two allelic frequency distributions, is satisfied when $\gamma^2 < p_{l.}p_{.l}\,\alpha_{0.05}$.

RESULTS

1. *Pooling occurs along both dimensions of the matrix of gametic frequencies: the case of association between alleles at two loci*

We have assumed six alleles at both loci (m=n=6) and examined the following types of pooling:

$P_1(1,1,1,1,2; S=0.75)$, $P_2(3,3; S=0.333)$, $P_3(5,1; S=0.231)$

Three allelic distributions were employed:

D_{I} : 0.168, 0.167, 0.167, 0.166, 0.166, 0.166

D_{II} : 0.45, 0.45, 0.03, 0.03, 0.02, 0.02

D_{III}: 0.75, 0.11, 0.05, 0.04, 0.03, 0.02

All three allelic distributions and all three types of pooling were applied to both loci. This resulted in 36 different combinations of allelic distributions and types of pooling. Each combination was tested at three levels of linkage correlation, determined by the range of γ^2. The three ranges were: 0.013–0.019, 0.0033–0.0043 and 0.008–0.001. We, therefore, examined a total of 108 cases. Each case was run either one hundred or two hundred times: cases of the type $P_i \times P_i$ were run one hundred times, cases of the type $P_i \times P_j$ two hundred times. The results are given in Figures 1 and 2.

Several points of interest emerge from Figure 1. First, the percentage, R, of reduced matrices which underestimate the amount of correlation is quite high. This is true for all cases except the ones in which four out of six alleles at each locus are electrophoretically identifiable. Polymorphisms with an electrophoretic resolution as high as this are expected to be rare (and combinations of two polymorphisms both of electrophoretic resolution as high as this even rarer) so we may conclude that in the great majority of the cases electrophoretic surveys

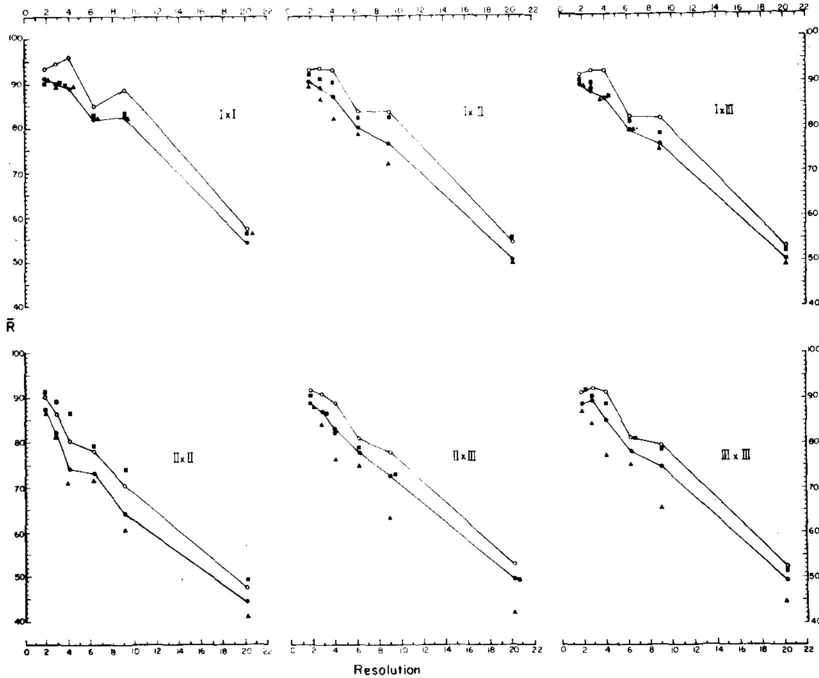

FIGURE 1.—The percentage ($\bar{R}$) with which pooling of alleles into electrophoretic classes results in underestimation of the degree of linkage disequilibrium. Abscissa: the combined degree of electrophoretic resolution ($S_{AB}=S_A \cdot S_B$) multiplied by mn, the product of the number of alleles at the two loci. Open marks: association is not detected at the 1% level of significance; solid marks: association is not detected at the 5% level of significance. Squares: low amount of linkage disequilibrium in the population ($0.001< \gamma^2 <0.008$); circles: intermediate amount ($0.003< \gamma^2 <0.004$); triangles: high amount ($0.013< \gamma^2 <0.019$). I, II and III refer to allelic distributions. Symbols are fully explained in METHODS.

underestimate the amount of linkage disequilibrium. Second, this percentage is rapidly increasing as the combined index, S_{AB}, of electrophoretic resolution decreases. In all likelihood the process is given by a sigmoid curve leveling off as S_{AB} approaches 0 and 1. Over much of its span and for the range of S_{AB} for which we are interested, the curve may approach linearity. It is interesting to note that the slope of this part of the curve is not affected by the allelic frequency distribution, D, or by the actual amount of linkage disequilibrium, γ^2. The slopes of the eighteen lines defined by the solid marks of Figure 1 are not statistically different, yielding -2.14 as the common estimate. This observation indicates that the components $S \times D$ and $S \times \gamma^2$ are not significant in determining the change in $\bar{R}$. But the intercept is affected by both D and γ^2. For the same combination of allelic frequency distribution, $D_i \times D_j$, $\bar{R}$ becomes lower the higher the value of γ^2, *i.e.*, the chances of not detecting an association because of imperfect discrimination of alleles become higher the weaker the association.

It is interesting to compare the effect of electrophoretic resolution on $\bar{R}$ with the

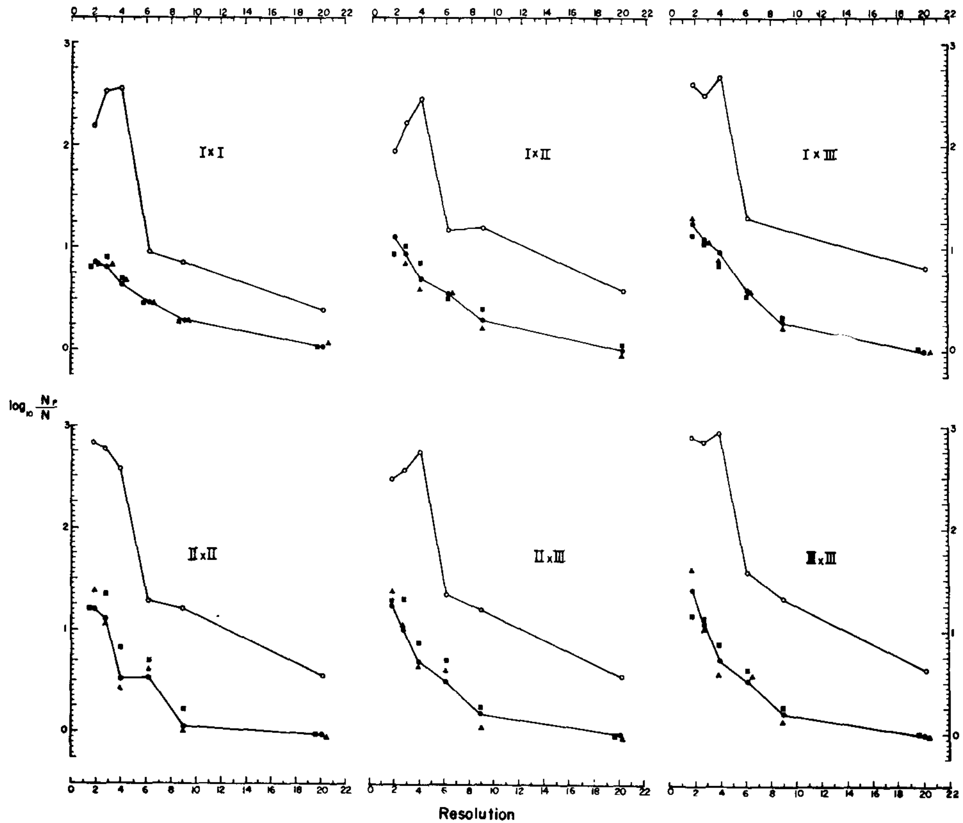

FIGURE 2.—Increase in sample size required so that the linkage disequilibrium is detected in 50% or 95% of the possible combinations resulting from the pooling of alleles into electrophoretic classes. N: sample size required for the detection of the association at the 5% level of significance when no pooling occurs; N_p: sample size required for the detection of the association at the 5% level of significance after pooling; open marks: the association is detected in 95% of the combinations; solid marks: the association is detected in 50% of the combinations; other notations as in Figure 1.

effect of the allelic distribution or the actual degree of correlation. The largest observed difference in the intercept of $\bar{R}$ as a result of change of allelic distributions was 10.03 and the largest observed difference as a result of change in the actual amount of correlation was 9.73. This difference in $\bar{R}$ can be compensated by changing the combined resolution index by 0.12 or by changing the electrophoretic resolution at each locus by one third ($0.12^{-0.5} = 0.346$).

In Figure 1 the solid marks give the frequency with which pooling underestimates the degrees of linkage disequilibrium when the requirement is that the correlation is detected at the 5% level of significance. In the same figure we give this same frequency when the requirement is that the correlation is detected at the 1% level of significance (open circles). As expected from the theory of the preceding section the number of correlations which are not detected because of

the imperfect allelic discrimination increased when the statistical requirement increased.

Figure 2 gives the increase in sample size required for the association to be detected at the 5% level of significance in 95% of the combinations of alleles (open circles) or in 50% of the combination of alleles (solid marks) resulting from a given type of pooling. For a degree of electrophoretic resolution lower than 0.5 (*i.e.*, $S_{AB} < 0.25$) sample sizes larger by 0.5 to 1.5 order of magnitude are required so that there would be an even chance that linkage correlation does not escape detection as a result of the imperfect discrimination of alleles. An increase of this level of assurance to 95% would require an increase in sample size by 1.5 to 3 orders of magnitude.

One might ask how much variance exists in R and N_p/N. Figures 3 and 4 give the distribution of R and N_p/N for 3 cases: one with the highest discrimination power ($D_{II} \times D_{II}$ pooled as $P_I \times P_I$), one with the lowest ($D_I \times D_I$ pooled as $P_3 \times P_3$), and one in between ($D_I \times D_{III}$ pooled as $P_1 \times P_3$). In addition, the first and the third case were among the ones with the largest variance in R and N_p/N,

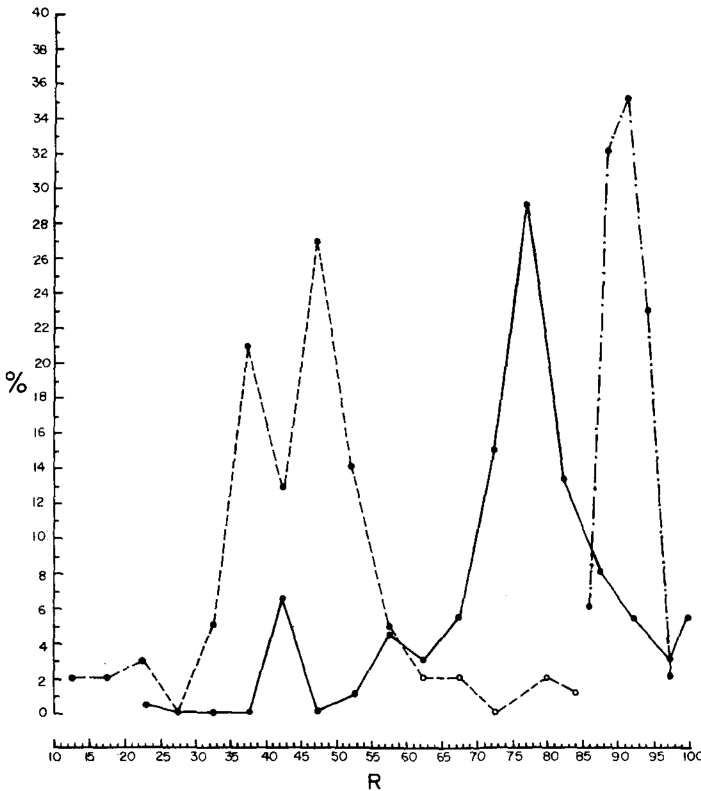

FIGURE 3.—The distribution among runs of the percentage of combinations in which linkage disequilibrium is detected at a level of significance lower than it is when discrimination of alleles is perfect. All three distributions are for $0.003 < \gamma^2 < 0.004$; dashes: case $D_{II} \times D_{II}$, $P_1 \times P_1$; solid: case $D_I \times D_{III}$, $P_1 \times P_3$; dashes and dots; $D_I \times D_I$, $P_3 \times P_3$.

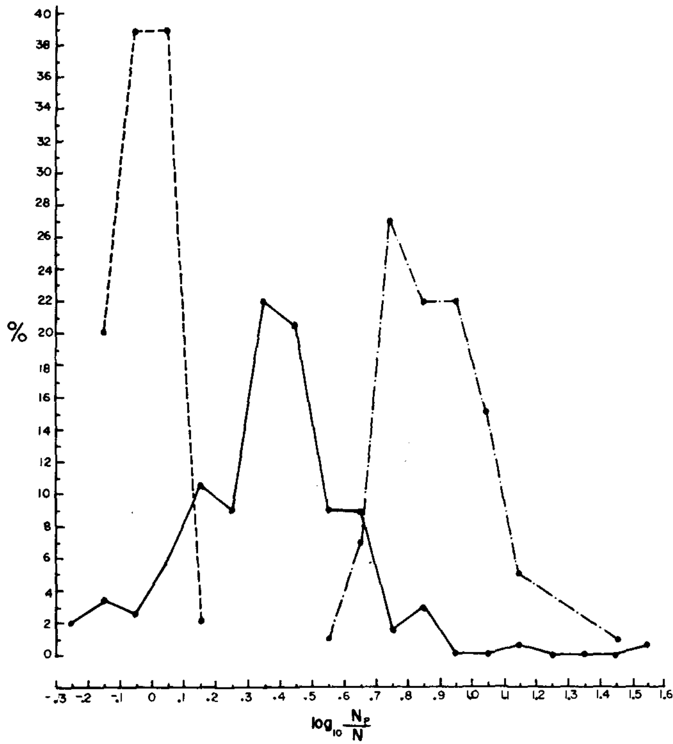

FIGURE 4.—The distribution among runs of the logarithm of the ratio of sample size required so that linkage disequilibrium is detected in 50% of the cases resulting from combining alleles in one class, to the sample size required for the detection of the disequilibrium when no combining occurs. The presented cases are the same as in Figure 3.

whereas the second case was one with the smallest variance. Most distributions of either R or N_p/N are unimodal and roughly symmetrical so that standard errors on the means are reliable. To obtain a better idea as to what extent the means are reliable, separate means were obtained for the first and for the second half of the runs in 12 cases. These means are given in Table 1. It can be seen that they are sufficiently close to each other and within the limits specified by the standard errors.

2. *Pooling occurs along one axis of the matrix of gametic frequencies: the case of association between inversions and allelozymes*

We have assumed three gene arrangements segregating in the population. This number was selected as the most representative of studies of linkage disequilibrium involving electrophoretic variation and inversions. Three sets of gene arrangement (or inversion) frequencies were considered: I_I: 0.334, 0.333, 0.333; I_{II}: 0.80, 0.10, 0.10; I_{III}: 0.75, 0.20, 0.05. Six alleles were assigned to the locus under consideration. The allelic frequency distributions and the types of pooling of alleles into electrophoretic classes were the same as in the previous section.

TABLE 1

Means of R *and* N_p/N *of two sets of runs involving two combinations of allelic frequency distributions and six types of pooling of alleles into electrophoretic classes*

Type of pooling	Number of runs	$D_{II} \times D_{III}$		$D_{III} \times D_{III}$	
		$\overline{R}$	$\log_{10}(\overline{N_p/N})$	$\overline{R}$	$\log_{10}(\overline{N_p/N})$
$P_1 \times P_1$	50	43.24	—0.037	44.08	—0.032
$P_1 \times P_1$	50	41.04	—0.051	44.98	—0.030
$P_1 \times P_1$	100	65.34	0.087	63.71	0.092
$P_1 \times P_2$	100	61.04	0.022	66.95	0.137
$P_1 \times P_3$	100	74.68	0.566	74.54	0.534
$P_1 \times P_3$	100	74.79	0.615	75.51	0.554
$P_2 \times P_2$	50	78.14	0.591	75.64	0.527
$P_2 \times P_2$	50	75.34	0.597	78.22	0.644
$P_2 \times P_3$	100	84.60	1.022	83.35	0.982
$P_2 \times P_3$	100	83.88	1.039	84.87	1.058
$P_3 \times P_3$	50	88.17	1.457	86.28	1.589
$P_3 \times P_3$	50	83.83	1.348	87.44	1.649

The 3 × 6 unreduced matrices of the frequencies of the inversion-allele combinations were generated in such a way as to give $0.013 < \gamma^2 < 0.019$.

The results are given in Table 2. In this table I_i stands for the inversion and D_i for the allelic frequency distribution. The rest of the notation is the same as in the previous section. It can be seen that the results are not drastically different from the ones obtained for the locus × locus case. Again, electrophoretic data will

TABLE 2

The frequency with which combining alleles results in lowering the detected level of significance of the association between alleles at a locus and inversions, and the increase in sample size required so that the association is detected in 50% of the allelic combinations

$I_i \times D_j$	$\overline{R}$			$\log_{10}(\overline{N_p/N})$		
	P_1	P_2	P_3	P_1	P_2	P_3
I × I	46.60(66.65)†	81.30(82.70)	84.00(86.65)	—0.007	0.365	0.470
I × II	43.20(62.76)	84.10(78.81)	84.67(82.76)	—0.032	0.616	0.734
I × III	42.13(65.04)	82.00(80.65)	82.33(85.05)	—0.031	0.283	0.711
II × I	45.93(62.76)	79.00(78.81)	81.33(82.76)	—0.006	0.423	0.515
II × II	42.67(56.62)	78.10(72.67)	80.50(76.62)	—0.031	0.315	0.683
II × III	38.93(60.62)	75.10(76.67)	80.00(80.62)	—0.031	0.207	0.793
III × I	50.93(65.04)	80.20(80.95)	85.00(85.05)	0.009	0.460	0.434
III × II	37.67(60.62)	72.00(76.67)	79.67(80.62)	—0.031	0.207	0.686
III × III	40.60(59.78)	76.90(75.83)	80.93(79.78)	—0.026	0.240	0.688

† The number in parenthesis is the value of $\overline{R}_{0.05}$ obtained from the regression line of Figure 1 that corresponds to the same $D_i \times D_j$ and to the same interval of γ^2.

underestimate the level of the association in the majority of the cases, except when the allelic resolution is high.

The numbers in parenthesis in Table 2 give the values of $\bar{R}$ that one could have obtained from the locus × locus case of the previous section. In the inversion × locus case the resolution, measured as mnS_AS_B (abscissa of Figure 1) is obtained by putting $m = 3$, $n = 6$, and $S_A = 1$. Assuming linearity as a first approximation, the expected values of $\bar{R}$ corresponding to different degrees of resolution can be obtained by using the appropriate regression lines defined by the solid marks in Figure 1. As mentioned earlier these lines have different intercepts but they yield −2.14 as the common estimate for the slope. It can be seen that the observed values of $\bar{R}$ are not very different from the "expected" ones when pooling occurs as P_2 and P_3, but are considerably higher when alleles are pooled as P_1 (*i.e.*, four out of six alleles are electrophoretically identifiable).

The fact that the risk of underestimating the amount of the association in the inversion × locus case is not much different from the risk of the locus × locus case (except when the electrophoretic resolution of the alleles at the locus is exceptionally high) suggests that this risk is affected by the combined rather than the separate indices of resolution. The more the index of one locus approaches zero, the more it affects S_{AB} and, therefore, $\bar{R}$ no matter what is the index of the other locus.

3. *Test on the sign of the disequilibrium over populations*

Here we have examined the possibility that the limitations in discrimination power obscure the consistency over populations in the sign of the correlation between the major alleles at two loci. Again, we assumed six alleles per locus and examined four allelic frequency distributions: one with the major allelic frequency being 0.7, another with 0.6, another with 0.5 and another with 0.4. The frequencies of the other five alleles were determined in such a way that the second most frequent allele was twice as frequent as the third, the third most frequent allele was twice as frequent as the fourth, and so on. The only type of pooling used here was the one in which three alleles form one electromorph and the other three alleles form another electromorph (P_2), and from the ten combinations of alleles resulting from this type of pooling only three were examined: the three most frequent alleles pooled together, the most frequent allele pooled with the third and fourth most frequent alleles, and the most frequent allele pooled with the two least frequent alleles.

For this section let $p_{1.}$ and $p_{.1}$ be the frequencies of the major alleles at locus $A(A_1)$ and $B(B_1)$, and $q_{1.}$ and $q_{.1}$ be the frequencies of the electromorphs containing A_1 and B_1. Our procedure was to generate a 6×6 matrix of gametic frequencies in the way described earlier, but with no restrictions on the actual degree of correlation (γ^2). The matrix was then reduced to 2×2 and the sign of the ratio D_1/D_2 was obtained, where $D_1 = p_{11} - p_{1.}p_{.1}$ and $D_2 = q_{11} - q_{1.}q_{.1}$. Each combination of allelic frequency distributions and way of pooling was run 200 times. We examined 36 such combinations. The relevant quantity here is how many times out of 200 the sign of D_1/D_2 is positive. Whether or not D_2 is

going to have the same sign as D_1 may be determined by a number of factors. We examined two of them: the relative size of $q_{1.}$ over $p_{1.}$ and the value of $D_1^2/p_{1.}p_{.1}$ which gives a measure of the correlation between the major alleles of the two loci.

In Figure 5 we plotted the percentage, F, in which D_1/D_2 was positive against $\frac{q_{1.}q_{.1} - p_{1.}p_{.1}}{q_{1.}q_{.1}}$. As expected, the two quantities are negatively correlated. The important observation here is that F rarely exceeds 70. This means that a large number of populations have to be surveyed in order to demonstrate consistency in the sign of the disequilibrium. Even if F is as high as 75 a minimum of 15 populations need to be examined in order to demonstrate a significant deviation from chance in the sign of the association. The actual degree of correlation does not seem to affect the probability of detecting the consistency in the sign of the correlation. To see this for each combination of allelic frequency distributions and way of pooling, we compared the one hundred runs with the lower $D_1^2/p_{1.}p_{.1}$ values to the one hundred runs with the higher values. In no case was the F from the first group of runs significantly lower than the F from the second group of runs. The degree of correlation between A_1 and B_1 does not affect F since a strong correlation precipitates an equally strong correlation of the opposite sign among

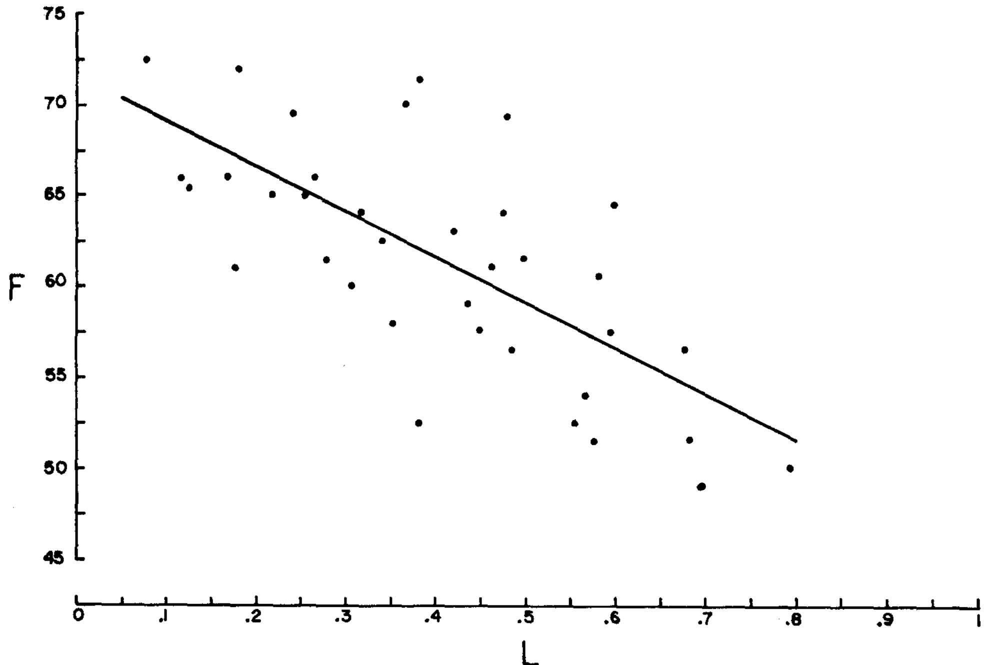

FIGURE 5.—The percentage (F) in which the sign of the correlation between the major alleles at two loci is the same as the sign of the correlation between the electromorphs that contain these alleles. $L=(q_{1.}q_{.1} - p_{1.}p_{.1})/q_{1.}q_{.1}$, where $p_{1.}$ and $p_{.1}$ are allelic frequencies, and $q_{1.}$ and $q_{.1}$ are electromorph frequencies.

the other alleles and the two correlations tend to cancel each other when pooling occurs.

DISCUSSION

The pooling of alleles into few detectable classes will not affect the test of random association among nonallelic genes only in the trivial case in which the correlation is zero. Selection, random drift or migration will always generate some correlation. It becomes, then, interesting to ask whether tests carried out on the combined classes of alleles generate a level of association consistently lower or consistently higher than the one that would have been detected otherwise. Our answer is that the cases in which such tests underestimate the strength of the association far outnumber those in which the strength is overestimated. This means that when the correlation is not very strong and/or when the sample size is small (cases that are very likely to occur in natural populations and in experimental practice) there is a high probability that linkage disequilibrium will escape detection.

This probability is higher the weaker the correlation and the more even the allelic frequency distributions. But it is affected much more drastically by the distribution of alleles into electrophoretic classes. In our simulations an average of 1.33 alleles per electrophoretic band (calculated as $1/S_A$) did not reduce the chances of detecting the disequilibrium. In fact, in 50.2% of all cases the pooling generated an association stronger and in 49.8% weaker than the one detected from the actual gametic frequencies. But when the average number of alleles per electromorph increased to 1.85, the detected amount of linkage disequilibrium was lower than the actual one 74.2% of the time. With 2 alleles per band this average was 78.8% and with 4.33 it was 89.6%.

At present we can only guess the average number of alleles per electromorph. If electrophoresis detects only those amino acid substitutions which involve change in net charge, then one can calculate from the genetic code that the detectable fraction of substitutions is approximately one-third (LEWONTIN 1974). Recently JOHNSON (1976) has shown that by varying gel pore size electrophoresis can detect an additional type of amino acid substitutions, those modifying the retardation of the protein by the gel. Using heat denaturation tests BERNSTEIN, THROCKMORTON and HUBBY (1973) showed that on the average 1.74 alleles per electromorph can be detected at the xanthine dehydrogenase locus of species of the *virilis* group of Drosophila. The same tests applied to octanol dehydrogenase revealed the presence of 2.6 alleles per electromorph (SINGH, HUBBY and THROCKMORTON 1975). In both studies only a small number of isofemale lines were subjected to only 4 to 6 categories of thermal liability. There is every reason to suspect that much more variability remains hidden. In the absence of evidence to the contrary one is forced to accept the opinion of KING and OHTA (1975) that these studies revealed "a second tip of the iceberg, and not the iceberg itself."

It follows that electrophoretic studies almost invariably have underestimated the amount of linkage disequilibrium in natural populations. In the light of the present analysis it is not surprising that in many cases correlations have not been

observed even between inversions and electromorphs of loci included in or closely linked to these inversions (see ZOUROS *et al.* 1974 for review). With a sample size of a few hundred or less, the chances of detecting the association are good only when the electrophoretic resolution at the locus is quite high or when the actual correlation is very strong. It becomes clear that we cannot conclude on the basis of the information now available that nonrandom associations between allelozymes and inversions or between allelozymes of two or more loci are rare events and, consequently, that linkage equilibrium is the rule in natural populations. The failure to observe frequent linkage disequilibria in sexually reproducing populations does not diminish the possibility that epistatic interactions among genes are important in evolution, and does not testify to the hypothesis of neutral protein variation, as OHTA (1973) and MUKAI, WATANABE and YAMAGUCHI (1974) have claimed. However, the lack of nonrandom associations between inversions and electromorphs can be justifiably used when hypotheses about differences in the amount of *electrophoretic* variation over enzyme classes are discussed (ZOUROS 1976).

LEWONTIN (1973) in his recent review of population genetics wrote that one of the most unambiguous ways of demonstrating that protein variation is under selection "is to find consistent linkage correlations among allozyme polymorphisms in natural populations. If loci are consistently correlated in a wide range of geographical localities, then they must be under selection. The chance of finding such correlations will remain small, however, until the genetic map of some organism is far better saturated with enzyme loci than is now the case." Our results strongly suggest that it would be difficult to detect the consistency of the correlation over many localities because of the limitations of electrophoresis. Clearly, a comprehensive study of linkage correlation should await not only the detailed genetic information about an organism, but also a technique of scoring gametic frequencies far more powerful than is presently available.

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LITERATURE CITED

BERNSTEIN, C. S., L. H. THROCKMORTON and J. L. HUBBY, 1973 Still more genetic variability in natural populations. Proc. Natl. Acad. Sci. U.S.A. **70**: 3928–3931.

CANNON, G. B., 1963 The effects of natural selection on linkage disequilibrium and relative fitness in experimental populations of *Drosophila melanogaster*. Genetics **48**: 1201–1216.

CHARLESWORTH, B. and D. CHARLESWORTH, 1973 A study of linkage disequilibrium in populations of *Drosophila melanogaster*. Genetics **73**: 351–359.

HILL, W. G., 1974 Estimation of linkage disequilibrium in randomly mating populations. Heredity **33**: 229–239.

JOHNSON, G. B., 1976 Hidden alleles at the α-glycerophosphate dehydrogenase locus in Golias butterflies. Genetics **83**: 149–167.

KING, J. L. and T. OHTA 1975 Polyallelic mutational equilibria. Genetics **79**: 681–691.

LANGLEY, C. H., Y. H. TOBARI and K. KOJIMA 1974 Linkage disequilibrium in natural population of *Drosophila melanogaster*. Genetics **78**: 921–936.

LEWONTIN, R. C., 1973 Population Genetics. Ann. Rev. Genet. **7**: 1-17. ——, 1974 *The Genetic Basis of Evolutionary Change.* Columbia University Press, New York.

MUKAI, T., T. K. WATANABE and O. YAMAGUCHI, 1974 The genetic structure of natural populations of *Drosophila melanogaster* XII. Linkage disequilibrium in a large local population. Genetics **77**: 771–793.

OHTA, T., 1973 Slightly deleterious mutant substitutions in evolution. Nature **246**: 96–97.

SINGH, R. S., J. L. HUBBY and L. H. THROCKMORTON, 1975 The study of genic variation by electrophoretic and heat denaturation techniques at the octanol dehydrogenase locus in members of the *Drosophila virilis* group. Genetics **80**: 637–650.

WALLACE, B., 1975 Gene control mechanisms and their possible bearing on the neutralist-selectionist controversy. Evolution **29**: 193–202.

ZOUROS, E., 1976 The distribution of enzyme and inversion polymorphism over the genome of Drosophila: evidence against balancing selection. Genetics **83**: 169–179.

ZOUROS, E. and C. B. KRIMBAS, 1973 Evidence for linkage disequilibrium maintained by selection in two natural populations of *Drosophila subobscura*. Genetics **73**: 659–674.

ZOUROS, E., C. B. KRIMBAS, S. TSAKAS and M. LOUKAS, 1974 Genic versus chromosomal variation in natural populations of *Drosophila subobscura*. Genetics **78**: 1223–1244.

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