STUDIES OF ENZYME EVOLUTION BY SUBUNIT HYBRIDIZATION

ROSS J. MACINTYRE
CORNELL UNIVERSITY

1. Introduction

Like many evolutionists in this era of molecular biology, I have been trying to assess the importance of natural selection at the molecular level.

Surely no one approach or single technique, or, in fact, no series of investigations which approaches the problem from just one direction will settle the dispute between the Darwinian and non-Darwinian camps. The final verdict will be pieced together from many experimental facts and theoretical insights. In this paper, I will present a progress report on a technique which will not by itself answer that outstanding question but which will, I hope, add to the evidence which will eventually decide the issue.

While initial results have been encouraging, there are still some outstanding questions. Specifically, does the technique measure what I think it does? And, do the differences in protein structure which it detects have any functional (and, thus, selective) significance?

2. Some background

Before I describe the technique, analyze the preliminary results, and discuss these questions, I will try to explain why I presently prefer the biochemical approach to the question of the importance of natural selection.

I began my research career as an experimental population geneticist interested in the adaptive significance of enzyme polymorphisms. My organism of choice was and still is, *Drosophila melanogaster*. However, I became rather profoundly pessimistic about my own or indeed anyone's ability to measure selective differences between the carriers of different electrophoretic variants of enzymes, or allozymes as they have been called (Prakash, Lewontin, and Hubby [17]). If, in fact, differences exist, we may not be able to measure them with present techniques. Why is this so? If one chooses to work with *Drosophila,* he may want to use population cages, the classical tool of the experimental population geneticist, to detect these selective differences in laboratory populations. Available evidence from population cages leads to a rather unsatisfactory conclusion, namely, if selective differences exist between different allozyme carriers, they must be very small.

For example, we have measured gene frequency changes in several allozyme systems of *D. melanogaster* and *D. simulans* over the last few years (MacIntyre and Wright [12] and O'Brien and MacIntyre [14]). In some instances, after the first few generations where complex chromosomal interactions may change single gene frequencies rather rapidly, a rather definite although slow change in gene frequencies may be seen. In others, no change is readily discernable. I have calculated selection coefficients in these cages under a simple model of selection against one homozygote. Table I shows the calculations and some of

TABLE I

SUMMARY OF GENE FREQUENCY ESTIMATES IN SEVERAL LONG TERM *Drosophila* POPULATION CAGES

The first seven cases are from MacIntyre and Wright [12] and the last two from O'Brien and MacIntyre [14].

$$\Delta\bar{p} = [s\bar{p}^2(1 - \bar{p}^2)]/(1 - s\bar{p}^2).$$

Cage	Enzyme	Number of generations	$\overline{\Delta p}$	s
3	esterase-6	28	.002	.017
4	esterase-6	28	.004	.03
5	esterase-6	27	.007	.05
6	esterase-6	30	.012	.11
7	esterase-6	32	.0007	.008
8	esterase-6	32	.002	.02
1	acid phosphatase-1	84	.0004	.003
1	leucine aminopeptidase-D	100	.003	.02

the background information. Data were taken from seven cages. In these populations, the number of generations was large enough to give us some confidence in the reality of any change in gene frequency on the one hand or in any equilibrium on the other. Three gene-enzyme systems were studied and in each, so-called "fast" and "slow" electrophoretic variants were the phenotypes used to determine gene frequencies.

In each cage, the mean change in allele frequency per generation is derived from p_0 (the "fast" allele frequency at apparent initial equilibrium) $-p_e$ (its frequency at end of the experiment)/number of generations and $\bar{p}$ (mean gene frequency of favored allele) is equal to p_0 (at initial apparent equilibrium) $+p_e$ (at end of experiment)/2. Finally, s (the selection coefficient) is calculated by rearranging the familiar formula in Table I. Note the very small selection coefficients ranging from 0.003 to 0.11. Both the reality of the gene frequency changes and the model of selection against only one homozygote may be properly questioned. What I want to do, however, is emphasize the very small changes in gene frequencies that characterize many population cage experiments with electrophoretic enzyme variants. We conclude simply that if the changes are

real, the selection coefficients are small. A recent and detailed study on a gene-enzyme system in *D. pseudoobscura* fully supports this conclusion (Yamazaki [19]).

But what does this imply with regard to meaningfully answering the question about the adaptive significance of enzyme polymorphisms in population cages? One thing it may mean is that each cage may represent a unique event and that results will not be repeatable. Laird and McCarthy have estimated that the haploid genome of *D. melanogaster* contains about 90,000 unique cistrons. Taking this at face value, and dividing by the approximately 300 map units of the genome, we get an estimate of 300 genes per map unit! While this may be an overestimate, there is no compelling reason to reject it out of hand (see also, Davidson and Hough [3]). It is fair to say, I believe, that most population geneticists have built their models with many fewer genes in mind. There is at least one implication in this that does not bode well for the investigator setting up his populations in order to study gene frequency changes at an allozyme locus. Remember that the selection coefficients associated with the genotypes are likely to be very small. Now, suppose there are 600 genes within one map unit of his locus. In order to define the problem, let us make the additional (and undoubtedly unrealistic) assumption that selection dictates that each of these 600 loci should be occupied by a wild type dominant allele. That is, at each there is selection for the wild type homozygote. Yet spontaneous mutations to deleterious alleles will have been occurring in the population. Each locus will in fact be polymorphic in the sense that rare mutant alleles will be present in low frequencies.

TABLE II

PROBABILITY OF SELECTING A "WILD TYPE" CHROMOSOME OVER A TWO MAP UNIT REGION UNDER SEVERAL MODELS OF SELECTION AT EVERY LOCUS AND A MUTATION RATE OF 1×10^{-6}

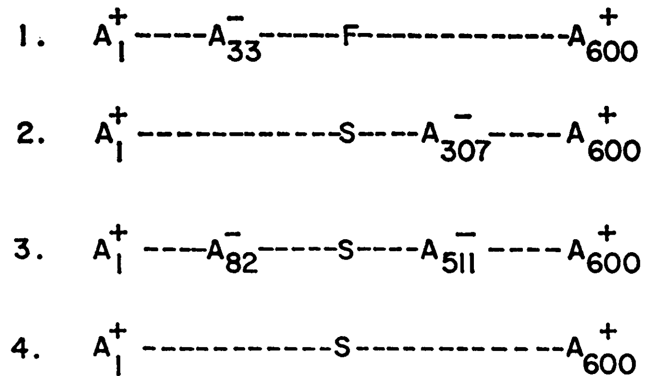
s	h	q when $\Delta q = 0$	Probability of selecting a wild type chromosome
0.1	0	3×10^{-3}	$(.997^{600}) = .17$
0.01	0	10^{-2}	$(.99^{600}) = .0024$
0.001	0	3×10^{-2}	$(.97^{600}) = 1.5 \times 10^{-7}$
0.1	0.1	10^{-4}	$(.9999^{600}) = .95$
0.1	0.01	10^{-3}	$(.999^{600}) = .55$
0.01	0.001	10^{-1}	$(.90^{600}) = 3.5 \times 10^{-28}$

Table II shows what some of these frequencies might be given a mutation rate per locus of 1×10^{-6}. The table is set up so that at each of the 600 loci, selection can act either against only the recessive homozygote or against both the recessive homozygote and the heterozygote. Column 3 in Table II contains the mutation-selection equilibrium frequency of the rare mutant allele in each situation. The frequencies, especially in the absence of selection against the

heterozygote, can be rather high. The important calculation is in the right hand column. This is the probability, given the various mutant allele frequencies at the 600 loci, that the investigator will choose a chromosome *free from mutant alleles* in the two map unit region when he chooses the founders for his experimental population. As you can see with selection coefficients associated with the deleterious mutant alleles of around 0.01, it is almost certain that he will choose chromosomes with some "genetic junk" closely associated with the allozyme locus. Note that each chromosome in the founders will almost always be unique with regard to its "genetic junk." Several investigations indicate that new deleterious mutations are associated with selection coefficients of 0.01 (for example, Kenyon [7]). Thus, if an investigator starts two populations each with different founders, the chromosomes may look like something I have drawn in Figure 1. In this figure it is assumed that the founders of a cage will contain four possibly different chromosomes. The area surrounding the allozyme locus represents one map unit on each side and is delimited by the number of genes (600) in the interval. Thus, in chromosome number one in cage one, the "fast" allele is linked to a mutant, deleterious allele at locus No. 33. The fourth chromosome for cage 2 has a "slow" allele linked to three different mutant genes. The dashes in Figure 1 simply indicate wild type alleles at the other loci. Notice that while the founders have the same initial frequencies of the "fast" allele of the enzyme locus, the other associated genes are very different.

In this model, I have assumed that the type of selection against each mutant allele at the 600 loci is the same, that is, the mutant alleles are completely recessive. This is, of course, quite unrealistic. In reality, if one can even think of selection acting in such an independent way, there may be selection against heterozygotes and mutant homozygotes at some loci, heterotic selection at others, and so on. The point I wish to make is that even in an oversimplified model, there can be tremendous variation in several samples of chromosomes. The rather variable results from duplicated experiments designed to detect single gene frequency changes in experimental populations, I think, support this idea.

Franklin and Lewontin [4] have put this concept in a sharper definition by equating the "unit of selection" as a correlated block of genes, that is, a small area of the chromosome in which the genes may be out of linkage equilibrium. Unfortunately, we have almost no experimental information about the extent of linkage disequilibria in natural populations. Nor are we, in my view, likely to be able to obtain meaningful data on this point in the near future. We simply don't have enough closely linked genes identified. Also, it is not very satisfying to have to replace the single locus, whose products we can directly examine, with the "correlated block" of genes whose boundaries may be always shifting and whose allelic contents may be largely unknown. The real danger here is that the gap between the new theory and feasible experimentation may be unbridgeable for the foreseeable future.



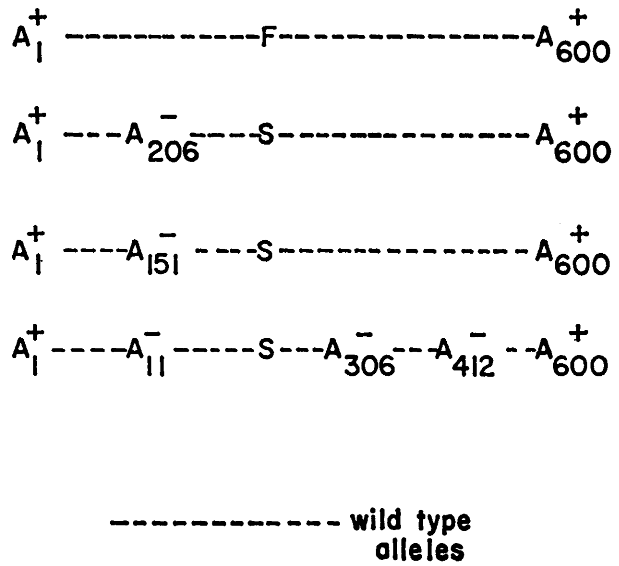


FIGURE 1

Chromosomes of hypothetical founders of two experimental populations. F and S refer to alleles specifying detectable electrophoretic variants of an enzyme. A_1–A_{600} refer to the 600 loci within one map unit of the gene controlling the allozymes.

Because of these considerations, I was left, in my own mind at least, with only one alternative—to approach the problem as a developmental biologist or a physiological geneticist rather than a population geneticist.

The basic difference between the neo- and non-Darwinians here can be simply stated: if allozymes or homologous enzymes in different species are selectively neutral, then they are functionally equivalent.

But how does one demonstrate the functional equivalence of two enzymes? One way is the so-called "shotgun" approach; that is, to measure and compare properties such as heat stabilities, inhibitor sensitivities, K_m's, and so on. But, if differences aren't found, it may simply be that the tests run weren't exhaustive, or if differences are found, they may not be relevant to the action of natural selection.

It is better, it seems to me, to proceed from that great truism of evolutionary biology, that natural selection acts on phenotypes and ask what is the phenotype of the enzyme? In this context, it must be its tertiary, and if it is an oligomeric enzyme, its quarternary structure. Thus, an enzyme must be folded properly to have a "wild type" active site or a "wild type" allosteric site or a "wild type" membrane attachment site. But, short of X-ray crystallography, how does one compare two enzymes with regard to their three dimensional configurations? Immunological tests are one way, but differences in antigenic sites may not always be accompanied by meaningful differences in enzyme structure.

In recent years, it has become evident that many, if not most enzymes, are composed of subunits (see Klotz and Darnall [8]). The bonding between these subunits is critical for the activity and perhaps the regulation of the activity of most of these enzymes (Cook and Koshland [2] and Iwatzuki and Okazaki [6]). It is, in fact, very fashionable as well as attractive to invoke subunit interactions in the control of cellular metabolism (Noble [13] and Haber and Koshland [5]). Hard evidence for this idea is rather sparse, however.

Nevertheless, studies on hemoglobin, the only protein for which the number and kind of amino acid residues involved in the quarternary structure are known, attest to the importance of this property in its basic function. Some 26 α chain amino acids contact the β chain and 27 amino acids in that latter subunit contact the α chain (Perutz, Miurhead, Cox, and Goaman [15]). So in each polypeptide chain almost twenty per cent of the amino acids are involved in the maintenance of a proper quarternary structure. The evolutionary conservatism of these amino acids is as pronounced as that of the amino acids involved in haem contact (see MacIntyre [11] for the details of this comparison). Furthermore, mutations in human hemoglobins affecting subunit interfaces, lead, in the majority of cases, to clinically detected anemias (Perutz and Lehmann [16]).

Thus, considering the all too meager definitive information from just one protein and much speculation, it can be stated at least as a working hypothesis that if differences in subunit affinities between allozymes or homologous enzymes can be demonstrated, then the enzymes should not be functionally equivalent.

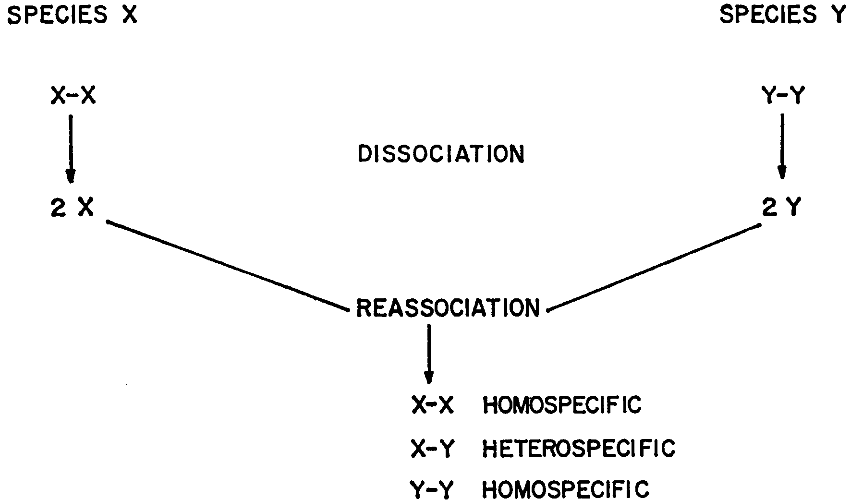


FIGURE 2

Outline of the experiments designed to detect interspecific differences in enzyme subunit affinities.

Observed homospecific:heterospecific ratio = enzyme activity of

$$[X\text{-}X + Y\text{-}Y]/(X\text{-}Y).$$

Expected homospecific:heterospecific ratio $= (p^2 + q^2)/2pq$,

where $p = [X\text{-}X + \frac{1}{2}(X\text{-}Y)]/[X\text{-}X + X\text{-}Y + Y\text{-}Y]$
and $q = [Y\text{-}Y + \frac{1}{2}(X\text{-}Y)]/[X\text{-}X + X\text{-}Y + Y\text{-}Y]$.

3. Experimental outline and details

In order to detect these differences, we have followed the simple rationale outlined in Figure 2. An enzyme from two species, which is assumed to be a dimer, is dissociated into its constituent subunits. These are mixed and the relative activities of the two homospecific (or homodimeric) and the heterospecific (or heterodimeric) enzymes are determined. The observed amounts can be expressed as an observed homospecific:heterospecific enzyme ratio, and compared to a ratio expected if subunit association is random. The data are frequently expressed as the *difference* between the two ratios, one observed and the other calculated.

At this point, and this will be discussed at greater length below, a difference implies either that the subunits do not randomly associate, or that the enzymes have different substrate turnover numbers.

We have made our most extensive tests to date with enzymes of different species because, initially, it wasn't clear if the method would distinguish be-

tween the undoubtedly more subtle allozymic differences. I will report only on the interspecific comparisons in this paper.

The enzyme I chose to work with is an acid phosphatase, by far the most prominent phosphatase after electrophoretic separation of extracts from *D. melanogaster* adults. The allozymic forms are shown in Figure 3. This is a starch gel pattern or zymogram developed for acid phosphatase activity of the extracts of four single flies (MacIntyre [12]). Flies monomorphic for "slow" and "fast" forms of the enzyme are designated as *AA* or *BB* homozygotes, respectively. The two heterozygous or *AB* flies in the middle of the gel show a three band pattern, characteristic of a dimeric enzyme (Shaw [18]). The gene had been mapped using electrophoretic variants shown in Figure 3.

We chose this enzyme for two reasons. First, there is ample interspecific variation in the electrophoretic mobility of this enzyme. Figure 4 shows the electropherogram of eight *Drosophila* species. *D. melanogaster* and *D. simulans* both have distinct allozymic forms of this enzyme. The enzymes from *D. emarginata* and *D. willistoni* have distinct subbands in acrylamide gels. This leads to some problems in the interpretation of the densitometrically delineated peaks of enzyme activity when the mixtures of subunits, after reassociation, are subjected to electrophoresis and staining. Many of these species are very closely related, for example, *D. willistoni* and *D. paulistorum*, *D. emarginata* and *D. sturtevanti*, and of course *D. melanogaster* and *D. simulans*. Their enzymes, however, are electrophoretically separable. This is fortunate since the quantitation of the homospecific and heterospecific enzymes involves either elution of

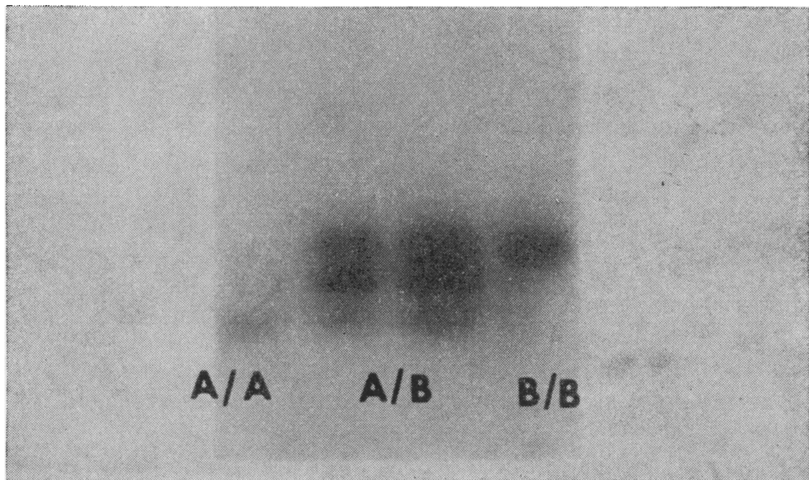


FIGURE 3

A starch gel with allozymic acid phosphatase patterns characteristic of "slow" or *AA* homozygotes, "slow"/"fast" or *AB* heterozygotes, and "fast" or *BB* homozygotes.

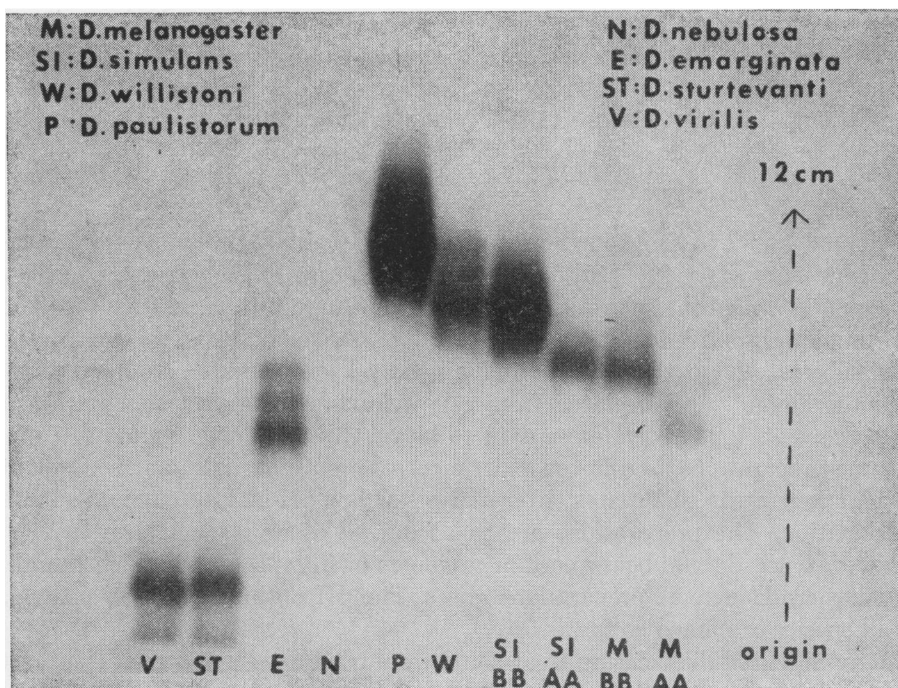


FIGURE 4

Acrylamide gel showing positions of the homospecific enzymes from eight *Drosophila* species. The *AA* and *BB* allozymes of *D. melanogaster* and *D. simulans* are also shown.

the precipitated dye from the gels or densitometry. On the other hand, the enzymes from the rather distantly related species *D. virilis* and *D. sturtevanti* are electrophoretically indistinguishable. Note that these prominent adult phosphatases are only assumed to be homologous with the one from *D. melanogaster*. Better evidence for this will be presented below.

The second reason this enzyme was chosen, was that in addition to the electrophoretic pattern of the heterozygote, we have other evidence indicating that this acid phosphatase is a dimer. This evidence will be published elsewhere (MacIntyre [9]) so I will simply summarize it here. First, the molecular weight of the enzyme from both *D. melanogaster* and *D. virilis*, the two most distantly related species in this survey, is 100,000, a value compatible with other enzymes known to be dimers (Klotz and Darnall [8]). Secondly, the subunit produced by the dissociating treatment we employ is almost exactly one half the size of the native enzyme. These subunits migrate to a single position in acrylamide gels where they reassociate to form active enzymes. This observation almost certainly rules out a heteromultimeric structure for the enzyme since it is improbable that the two different subunits would have the same net charge

and size. Finally, we have chemically induced 15 mutations which eradicate or alter the activity of the enzyme. A complementation analysis with all 15 mutants produced results entirely compatible with the interpretation that a single structural gene codes for this enzyme (Bell and MacIntyre [1]).

4. Preliminary experiments

I do not intend to go into extensive detail about certain preliminary information. (See MacIntyre [9], [10] for the details.) We assay the enzyme's activity by coupling α-naphthol phosphate with the diazonium salt, Fast Red TR. The red complex is soluble in detergent and glacial acetic acid. Thus, we can use the same substrate for both test tube and gel assays. Furthermore, we have correlated the amount of dye eluted from gels with densitometric tracings. Use of the latter technique allows more data to be taken in a shorter period of time. Enzyme preparations used in the experiments are only partially purified, about ten fold over crude supernates, after dialysis at low pH and ammonium sulfate fractionation. The preparations are free from all other phosphatase activity, however. I will discuss below certain problems of interpretation which the use of rather crude enzyme preparations raises. The pH optimum for the enzyme, for all species examined so far, is 5.1.

Many accepted dissociating treatments were tried on the enzyme. The most effective, in terms of good recovery of enzyme activity, is exposure of the enzyme to high pH levels. Figure 5 shows the inactivation which takes place over rather narrow pH intervals. The inactivation profiles of the enzymes from three species *D. melanogaster*, *D. virilis*, and *D. simulans* are shown in this figure. Dilute NaOH was added to extracts and the pH was recorded. Aliquots were assayed for enzyme activity at various pH's. The data are plotted in terms of per cent of control activity remaining *versus* pH. The arrows at the top of this figure indicate the pH at which complete inactivation just occurs for each species' enzyme. Thus, the acid phosphatase from *D. melanogaster* is completely inactivated at pH 10.7, *D. simulans*' enzyme at pH 10.9 and *D. virilis*' at 11.0. These differences are repeatable. I should point out that care must be taken not to raise the pH too far beyond the level at which inactivation is complete. If the pH is raised too high, too little enzyme activity is regained after adjustment of the pH back to neutrality.

From 20 to 85 per cent of the initial activity can be regained if the pH inactivated preparations are dialyzed back against Tris-maleate buffers. Curiously enough, the enzymes from different species may differ in the pH optimum for reassociation. This is shown in Figure 6. In this experiment, aliquots of completely inactivated extracts were dialyzed against Tris-maleate buffers of pH's ranging from 4.9 to 8.2. The activity regained by each aliquot, here expressed as optical density at 540 millimicrons, was then plotted against the pH of the buffer against which it was dialyzed. Note that the three species' enzymes have different pH optima for reassociation.

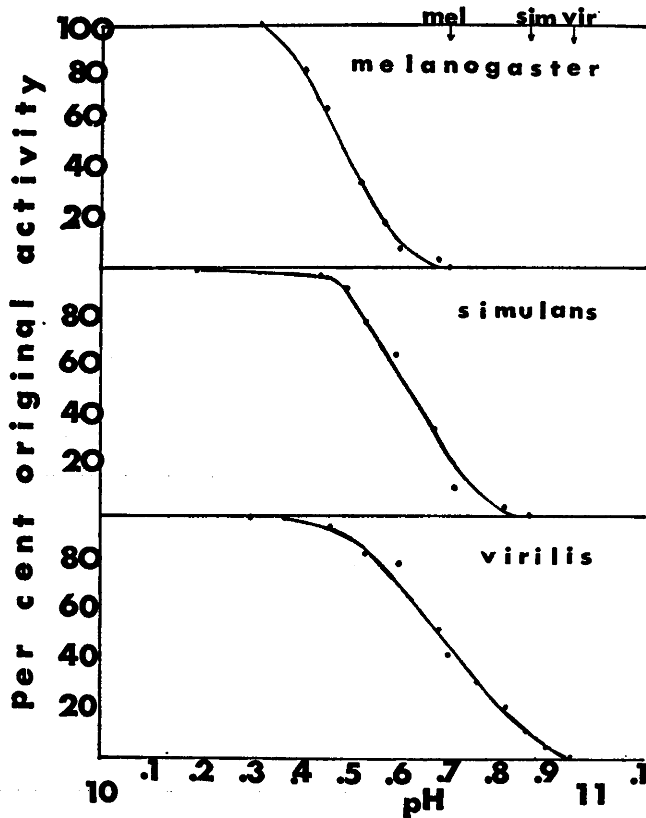


FIGURE 5

Inactivation curves of acid phosphatase-1 from *D. melanogaster*, *D. simulans* and *D. virilis*.

0.1 N NaOH was added to 5.0 ml of a partially purified enzyme preparation in 0.05 M NaCl at 25°C. After mixing, the pH was measured with a combination microelectrode. At the pH's indicated on the graph, 0.2 ml aliquots were removed and immediately assayed for acid phosphatase activity. Activities were corrected for the volume of NaOH added. The pH at which 100 per cent inactivation occurs for each species' enzyme is indicated on the top line.

In several preliminary subunit hybridization experiments with enzymes from *D. melanogaster*, *D. virilis*, and *D. simulans*, we tested for several things: (1) Does exposure to high pH dissociate the enzyme? (2) Do the subunits reassociate in all possible combinations during dialysis against the Tris-maleate buffers? (3) Can the electrophoretic patterns of the reassociated enzymes be reliably quantitated? (4) Are the results repeatable? Figure 7 shows that in the three tests, the enzyme dissociates at high pH and the subunits do reassociate

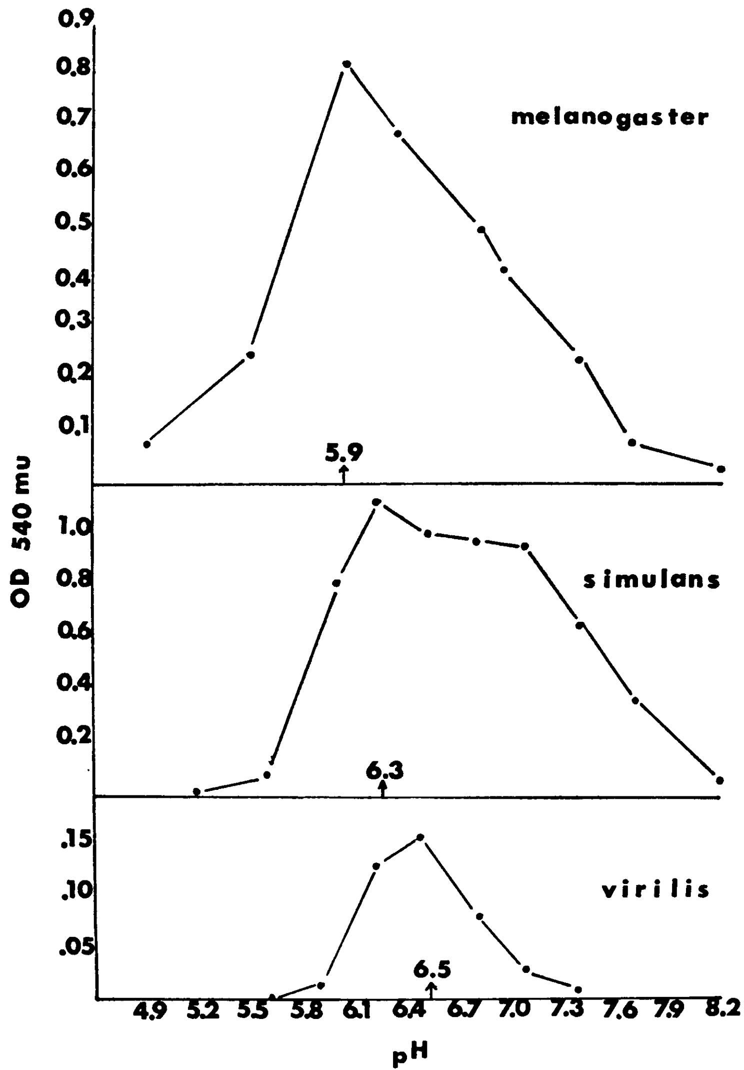


FIGURE 6

pH optima for reassociation of subunits of acid phosphatase-1 from *D. melanogaster*, *D. simulans* and *D. virilis*.

2.0 ml aliquots of pH inactivated acid phosphatase-1 from each of the three species (*D. melanogaster*; pH 10.7, *D. simulans*, pH 10.8, *D. virilis*, pH 11.0) were dialyzed against 0.4 Tris-maleate buffers at the indicated pH's for 72 hours at 4°C. The aliquots were then assayed for acid phosphatase activity. Apparent pH optima for each species are indicated on the abscissae.

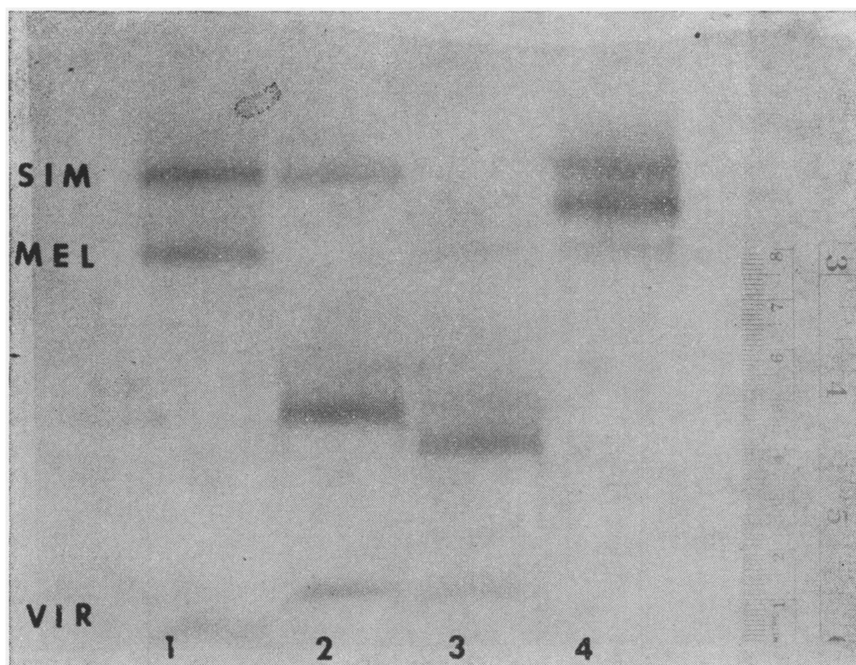


FIGURE 7

Electropherogram of acid phosphatase-1 from *D. virilis* (VIR), *D. simulans* (SIM) and *D. melanogaster* (MEL)(slot 1), the reassociated subunits from *D. simulans* and *D. virilis* (slot 2), from *D. melanogaster* and *D. virilis* (slot 3), and *D. melanogaster* and *D. simulans* (slot 4). In each mixture, the heterospecific enzyme is in the middle of each pattern.

to form *active* heterospecific enzymes during dialysis against the Tris-maleate buffer. In slot 1, the positions in the gel of the undissociated homospecific enzymes for each of the three species are shown. Above slot 2 is the pattern, after electrophoresis, of the reassociated mixture of subunits from *D. simulans* and *D. virilis*. The intermediate zone of activity represents the heterospecific enzyme. Slots 3 and 4 show the patterns of reassociated subunits from *D. melanogaster* and *D. virilis* (slot 3) and *D. melanogaster* and *D. simulans* (slot 4). Note that a heterospecific enzyme formed in each mixture of subunits from two different species. The presence of the active heterospecific enzyme almost certainly establishes the homologies between the enzymes. In Table III are the detailed quantitative data for one of the tests, *D. virilis* and *D. melanogaster*. Each experiment was repeated and the subunits reassociated at each species' pH optimum for reassociation, that is, at pH 5.9, the pH optimum for reassociation of the enzyme from *D. melanogaster* and at pH 6.5, the pH optimum for the *D. virilis* enzyme. The fourth column indicates how much activity was

TABLE III

RESULTS OF EXPERIMENTS INVOLVING REASSOCIATION OF ACID PHOSPHATASE-1 SUBUNITS OF *D. melanogaster* AND *D. virilis*

Ranges for the per cent of total acid phosphatase in each zone and for the homospecific:heterospecific ratios are given in parentheses. Number of determinations: experiment 1 (pH 5.9)-8, (pH 6.5)-7; experiment 2 (pH 5.9)-5, (pH 6.5)-4. The expected ratio is calculated as follows: p (*mel* subunits) = mean proportion of *melanogaster* homospecific enzyme + $\frac{1}{2}$ mean proportion of heterospecific enzyme; q (*virilis* subunits) = $1 - p$; expected ratio = $(p^2 + q^2)/2pq$. For example, in experiment 1 at pH 5.9: p (*melanogaster* subunits) = $(9 + \frac{1}{2}70)/100 = 0.44$; q (*virilis* subunits) = $1.00 - 0.44 = 0.56$; expected ratio = $[(0.44)^2 + (0.56)^2]/2(0.44)(0.56) = 1.00$.

pH of reassociation	Experiment no.	Subunits	Per cent reactivation after dialysis	Per cent of total acid phosphatase in each zone			Homospecific:heterospecific ratio	Expected ratio	Difference
				Melanogaster homospecific	Heterospecific	*Virilis* homospecific			
5.9	1	*melanogaster* *virilis* mixture	31 34 52	9 (7–11)	70 (67–76)	21 (17–22)	0.43 (0.32–0.49)	1.00 (1.00–1.04)	−0.57 (0.51–−0.68)
5.9	2	*melanogaster* *virilis* mixture	33 35 29	12 (11–13)	70 (68–74)	18 (15–19)	0.42 (0.35–0.47)	1.00 (1.00)	−0.58 (−0.53–−0.65)
6.5	1	*melanogaster* *virilis* mixture	22 49 38	10 (9–12)	67 (64–69)	23 (21–25)	0.50 (0.45–0.56)	1.00 (1.00–1.04)	−0.50 (−0.44–−0.57)
6.5	2	*melanogaster* *virilis* mixture	22 47 30	9 (8–10)	71 (70–71)	20 (19–22)	0.41 (0.41–0.43)	1.00 (1.00)	−0.59 (−0.59–−0.61)

regained, both by subunits from one species alone and when mixed in equal proportions. The relative activities of the three zones in the electrophoretic pattern of the reassociated mixture of subunits are in the next three columns. The ranges for each set of determinations (usually from 4–8) are in parentheses. If experiment number 1 (at the reassociation pH of 5.9) is taken as an example, then the average relative activity of the *D. melanogaster* homospecific enzyme is 9 per cent. The *D. virilis* homospecific enzyme contributes 21 per cent to the total. The heterospecific enzyme activity represents 70 per cent of the activity in the reassociated mixture of subunits. The observed homospecific:heterospecific activity ratio in this example is $(9 + 21)/70$ or 0.43. At the bottom of the table, the expected ratio for this particular experiment is calculated. The frequency of *D. melanogaster* subunits which reassociated to form active enzymes is $(9 + \frac{1}{2}70)/100$ or 0.44. The frequency of effectively reassociating *D. virilis* subunits, then, is $1 - 0.44$ or 0.56. The expected ratio is then equal to $(0.56)^2 + (0.44)^2/2(0.44)(0.56)$ or approximately 1. Then, finally, the difference between the observed and expected ratio in this example is $0.43 - 1.00$ or -0.57.

TABLE IV

SUMMARY OF RESULTS FROM ACID PHOSPHATASE-1 SUBUNIT REASSOCIATION EXPERIMENTS INVOLVING *D. melanogaster*, *D. simulans*, AND *D. virilis*

The ratios are the results of two experiments for each interspecific test.

Interspecific test	Reassociated at pH	Observed homospecific:heterospecific enzyme ratio	Difference from expected ratio
melanogaster	5.9	(1) .43 (2) .42	−.57 −.58
×			
virilis	6.5	(1) .50 (2) .41	−.50 −.59
simulans	6.3	(1) .60 (2) .79	−.40 −.21
×			
virilis	6.5	(1) .79 (2) .75	−.21 −.25
melanogaster	5.9	(1) 1.44 (2) 1.38	+.44 +.38
×			
simulans	6.3	(1) 1.50 (2) 1.33	+.42 +.33

Table IV summarizes the results for the three pairwise tests involving *D. melanogaster*, *D. virilis*, and *D. simulans*. Reported in this table are both the observed homospecific:heterospecific ratios and the differences of these from the ratios expected if subunit association were random and enzyme activities were equal. The correspondence between the duplicate experiments is good in

every case but that one involving *D. virilis* and *D. simulans* at a reassociation pH of 6.3. We have repeated this test several more times, however, and more consistently find a difference of about −0.30. Note the independence of the results from the pH optimum of reassociation. The data are expressed ultimately as a difference between observed and expected ratios. The sign of the difference is important. A minus indicates more than expected heterospecific enzyme activity is present. A plus sign indicates more than expected homospecific enzyme activity has been measured.

The use of *D. melanogaster* and *D. simulans* allowed us to directly test another assumption implicit in the methodology, namely, that the methods used in purification, dissociation, and reassociation do not impair the functional integrity of the subunits. Specifically, with these two species we can make several *in vitro versus in vivo* comparisons, since they both have electrophoretic variants and will form viable interspecific hybrids. Thus, we determined the differences between observed and expected homospecific:heterospecific ratios in three tests and compared them with those obtained from the corresponding interspecific hybrids or intraspecific heterozygotes. The data are summarized in Table V.

TABLE V

COMPARISON OF *in vitro* AND *in vivo* RESULTS WITH REGARD TO REASSOCIATION OF ACPH-1 SUBUNITS

Difference between observed and expected homospecific/heterospecific ratio ± s.d.

Test	Difference ± S.D.	Probability
(A) *D. melanogaster*		
AA × *BB* (*in vitro*)	+0.31 ± 0.20	$t = 0.48$
Acph-1^A/*Acph-1*^{B} heterozygotes (single flies)	+0.22 ± 0.19	$P > 0.5$
(B) *D. simulans*		
AA × *BB* (*in vitro*)	+0.23 ± 0.06	$t = 1.96$
Acph-1^A/*Acph-1*^{B} heterozygotes (mass homogenates)	+0.32 ± 0.12	$0.10 > P > 0.05$
(C) Interspecific hybrids		
BB (*melanogaster*) × *BB* (*simulans*) (*in vitro*)	+0.32 ± 0.03	$t = 0.22$, $P > 0.5$ (vs. *D. melanogaster* ♀♀ × *D. simulans* ♂♂); $t = 2.22$, $0.05 > P > 0.01$ (vs. *D. simulans* ♀♀ × *D. melanogaster* ♂♂)
D. melanogaster ♀♀ × *D. simulans* ♂♂ (single flies)	+0.33 ± 0.12	
D. simulans ♀♀ × *D. melanogaster* ♂♂ (single flies)	+0.28 ± 0.04	

In the first case, the electropherograms of *D. melanogaster* heterozygotes for the alleles specifying the "slow" and "fast" electrophoretic variants were compared with the pattern obtained when partially purified, dissociated and reassociated "slow" and "fast" forms of the enzyme were used as the sources of the mixed subunits. In the second case, *D. simulans* heterozygote patterns are

compared in a similar way with the corresponding dissociated and reassociated "slow" and "fast" enzymes from this species. Also, reciprocal interspecific hybrids are compared with the *in vitro* results from the *D. melanogaster* × *D. simulans* test. The results agree well in every case except one, but this is close to the acceptable level. The *in vitro* results appear to faithfully reflect *in vivo* enzyme subunit associations.

5. Interspecific tests

The initial tests with *D. melanogaster*, *D. simulans*, and *D. virilis* gave me enough confidence to go ahead and survey several selected species from the genus.

TABLE VI

SUMMARY OF THE PHYLOGENETIC RELATIONSHIP AND PRELIMINARY INFORMATION ABOUT THE ACID PHOSPHATASE-1 ENZYMES FROM THE ELEVEN SPECIES USED IN THE SUBUNIT HYBRIDIZATION TESTS

The asterisk indicates that the *BB* enzyme of *D. melanogaster* = 1.00. *Simulans*, *melanogaster*, and *nebulosa* have electrophoretic variants.

Species	Species group	Subgenus	Electrophoretic position*	pH optimum	pH of dissociation	pH optima for reassociation
D. melanogaster	*melanogaster*	*Sophophora*	0.83, 1.00, 1.07	5.1	10.3–10.7	5.9
D. simulans	*melanogaster*	*Sophophora*	1.03, 1.17, 1.31	5.1	10.5–10.9	6.2
D. willistoni	*willistoni*	*Sophophora*	1.24	5.1	10.8–11.2	6.4
D. paulistorum	*willistoni*	*Sophophora*	1.38	5.1	10.8–11.2	6.9
D. nebulosa	*willistoni*	*Sophophora*	1.41, 1.69	5.1	10.2–10.7	6.5
D. emarginata	*saltans*	*Sophophora*	0.76	5.1	10.2–10.7	6.5
D. sturtevanti	*saltans*	*Sophophora*	0.28	5.1	10.3–10.9	6.1
D. virilis	*virilis*	*Drosophila*	0.28	5.1	10.4–11.0	6.5
D. mulleri	*repleta*	*Drosophila*	0.28	5.1	10.0–10.7	6.9
D. mercatorum	*repleta*	*Drosophila*	0.28	5.1	9.9–10.6	6.7
D. immigrans	*immigrans*	*Drosophila*	0.69	5.1	10.2–10.8	7.2

Table VI summarizes the phylogenetic relationships of the species and necessary preliminary information about each which must be obtained before the experiments can be conducted. Eleven species which were used in all the possible pairwise tests are listed in the left column of the table. The relative electrophoretic positions of the homospecific enzymes (and allozymes where these have been found) are given in column 4. The "fast" or *BB* form of the *D. melanogaster* enzyme is set at 1.00. Note the interspecific variation in the pH inactivation interval and in the pH optimum for reassociation. In Figures 8 and 9 are the gel patterns obtained for some of the possible tests, that is, those tests in which the homospecific and heterospecific enzymes can be well enough

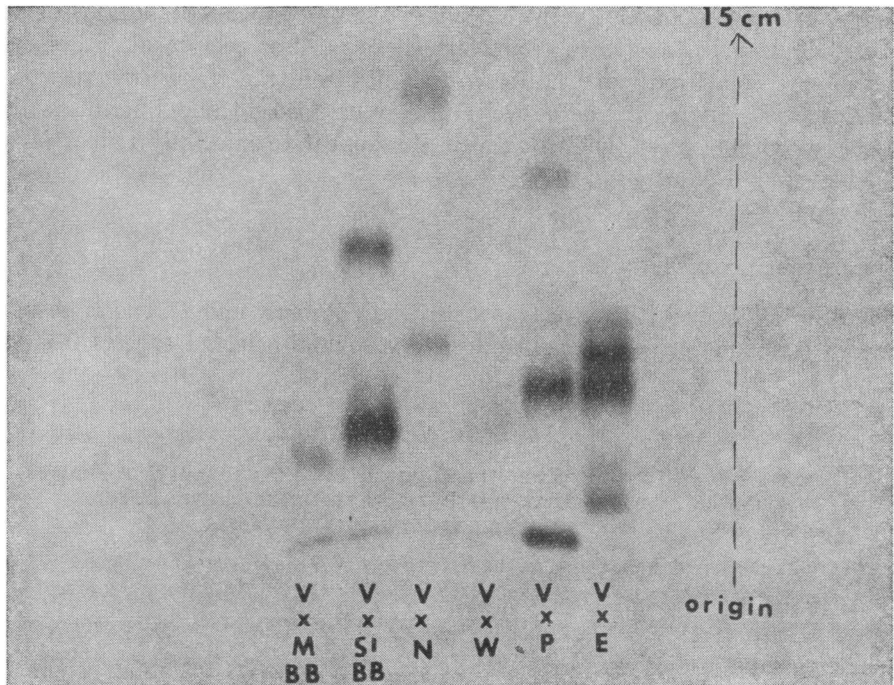


FIGURE 8

Acrylamide gel showing the six patterns of homospecific and heterospecific enzymes which formed during reassociation of subunits from the *D. virilis* acid phosphatase with subunits from the enzymes of six other *Drosophila* species. See Figure 4 for species designations.

separated by electrophoresis for an adequate densitometric analysis. Figure 8 shows eight tests with *D. virilis* as one common species. It can be seen in both figures, that in every case, a heterospecific enzyme forms, indicating that these are homologous enzymes. The subbanding in some patterns is also evident, but these can be correctly resolved if electrophoresis is carried out for a long enough time.

As you might expect, the quantitative data are massive. Figures 10 and 11 show them in graphic form. Given for each test is the mean difference between the observed and expected homospecific:heterospecific ratio and the 95 per cent confidence interval. Six to ten determinations were made on each test. Note that if the 95 per cent confidence interval overlaps the line of zero in the center, either subunit association is random or the enzymes have equal activities. In tests which fall on the right side of the line, the homospecific enzymes are preferentially formed or are more active. On the left side of the line, the heterospecific enzyme is disproportionally represented. The species pairs are roughly arranged, top to bottom, with increasing phylogenetic distance.

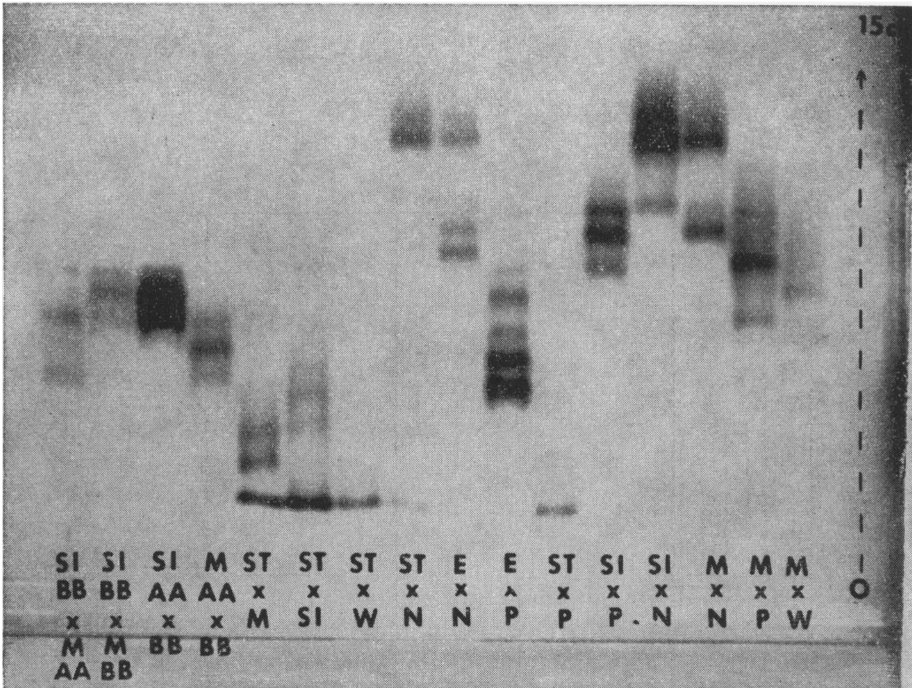


FIGURE 9

Acrylamide gel showing all possible subunit reassociation patterns involving the following species: *D. melanogaster* (M) with *AA* and *BB* allozymes, *D. simulans* (SI) also with *AA* and *BB* allozymes, *D. willistoni* (W), *D. paulistorum* (P), *D. nebulosa* (N), *D. emarginata* (E) and *D. sturtevanti* (ST).

The general conclusion to be drawn from these results is that there is variation between the homologous enzymes of closely related species that is detectable with the technique of subunit hybridization.

Many other observations can be made from the data presented in Figures 10 and 11. First of all, the intraspecific tests and the interspecific tests between species in the same species groups, without exception, have differences that indicate preferential formation or activity of the homospecific enzymes. In tests between species groups, however, both plus and minus differences were found. The meaning of this is not evident.

Secondly, the test appears to be extremely sensitive. A selected group of data was abstracted in Table VII. Reported here are the differences between observed and expected ratios in the tests involving *D. virilis* and *D. sturtevanti* with six other species from the subgenus *Sophophora*. Note that the enzymes of *D. melanogaster* and *D. simulans* are clearly different both in the tests with *D. virilis* and in the tests with *D. sturtevanti*. On the other hand, the enzymes from

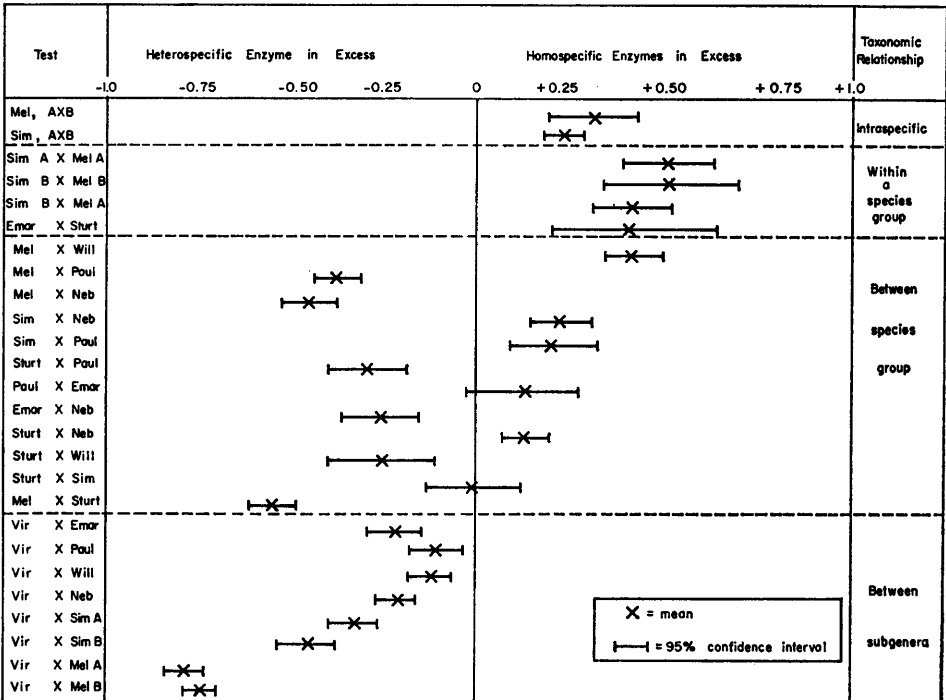


FIGURE 10

Differences of observed homospecific:heterospecific acid phosphatase activity ratios from those expected if subunit association were random, including all possible subunit hybridization tests involving the species listed in Figures 8 and 9. Code: Mel = *D. melanogaster*; Sim = *D. simulans*; Emar = *D. emarginata*; Sturt = *D. sturtevanti*; Will = *D. willistoni*; Paul = *D. paulistorum*; Neb = *D. nebulosa*; Vir = *D. virilis*.

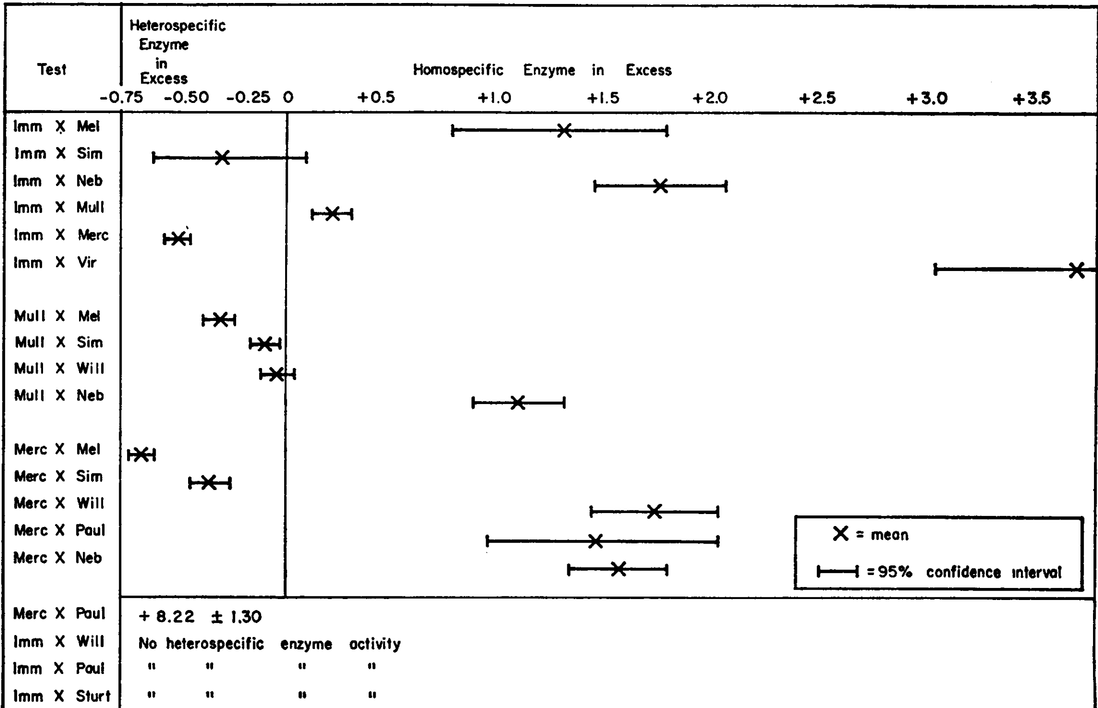


FIGURE 11

Differences of observed homospecific:heterospecific acid phosphatase activity ratios from those expected if subunit association were random, including all possible subunit hybridization tests involving *D. mulleri*, *D. mercatorum*, and *D. immigrans* with themselves and the other eight species listed in Table VI. Code: Imm = *D. immigrans*; Mull = *D. mulleri*; Merc = *D. mercatorum*; see Figure 10 for other species.

TABLE VII

RESULTS OF TESTS INVOLVING *D. virilis* AND *D. sturtevanti*
DIFFERENCE OF OBSERVED HOMOSPECIFIC:HETEROSPECIFIC RATIO FROM THAT EXPECTED IF SUBUNIT ASSOCIATION WERE RANDOM

Standard errors are shown with ± sign. Only *D. simulans* compared with *D. sturtevanti* is not significantly different from zero.

	D. virilis	*D. sturtevanti*
D. melanogaster	−0.74 ± .02	−0.54 ± .02
D. simulans	−0.46 ± .03	−0.01 ± .05
D. willistoni	−0.12 ± .02	−0.25 ± .06
D. paulistorum	−0.11 ± .03	−0.29 ± .04
D. nebulosa	−0.22 ± .02	+0.12 ± .03
D. emarginata	−0.23 ± .03	+0.42 ± .08

D. paulistorum and *D. willistoni,* two closely related species, are not differentiated by either test. Yet, their enzymes are separable by electrophoresis. *D. nebulosa,* a close relative of *D. willistoni* and *D. paulistorum* does appear to have an acid phosphatase which shows quite different reassociation patterns. Even more striking is the difference between the electrophoretically identical enzymes of *D. sturtevanti* and *D. virilis.* In every test except the one with *D. willistoni,* these two enzymes respond differently.

A third point to be made from the data in Figures 10 and 11 is that no two enzymes, when all possible pairwise tests are considered, are unequivocally the same in the properties measured by this technique. Note especially the differences between the enzymes of *D. mulleri* and *D. mercatorum.* Even the enzymes from *D. willistoni* and *D. paulistorum,* which appear to be the most similar, give very different results when tested with the dissociated enzyme from *D. melanogaster.* Finally, the subunits from *D. virilis* in almost every test preferentially associate (or form more active enzymes) in heterospecific combinations. On the other hand, the subunits from *D. immigrans* show little, if any, tendency to hybridize with subunits from the enzymes of different species. A complete analysis of these data will be published elsewhere.

6. Discussion

Some mention must be made at this point of the still unanswered questions about this method of detecting evolutionary changes in homologous enzymes. One obvious problem is that the proteins are not pure. In other words, could extrinsic factors and not the structures of the subunits be responsible for these patterns? Obviously, only with pure preparations can this problem really be solved. However, we have further purified the enzymes from *D. melanogaster* and *D. virilis* to about 80 fold by phosphocellulose chromatography. The very same homospecific:heterospecific enzyme ratio is obtained when these substantially purer preparations are used as the source of the subunits. In addition, we have obtained uncontaminated preparations of just the *D. melanogaster*-

D. virilis heterospecific enzyme. When it is dissociated and the subunits allowed to reassociate, both the two homospecific and the heterospecific enzymes form. The homospecific:heterospecific enzyme ratio in this experiment is again exactly the same as the ratio obtained when *D. melanogaster* and *D. virilis* homospecific enzymes are dissociated. (See MacIntyre [9] for details.)

If the differences between observed and expected ratios are due to intrinsic properties of the subunits, that is, differences in amino acid sequences, then another unanswered question arises. Specifically, do the differences reflect changes in amino acids affecting subunit interfaces or the enzymatic activity of the protein? Thus, in the test of *D. melanogaster* and *D. virilis* subunits, the homospecific:heterospecific ratio was in the range 0.3 to 0.4. Does this mean almost three times as much of the *D. melanogaster-D. virilis* heterospecific enzyme formed during reassociation? Or perhaps the expected amount forms but this novel phosphatase might turn over three times as many molecules of substrate as either homospecific enzyme.

In these experiments, there have not been measurable amounts of protein in the electrophoretically separated zones of acid phosphatase activity. We could, of course, start with pounds of flies rather than grams in the initial extraction of the enzyme, but as long as the preparations are only partially purified, one cannot be sure that measurements of specific activities would provide definitive information. We have some indirect evidence which suggests, in the *D. melanogaster* × *D. virilis* test, that the homospecific:heterospecific enzyme ratio reflects actual amounts of enzyme formed during subunit reassociation. Specifically, (1) the K_m's of all three enzymes are identical, and (2) the initial rate of increase of enzyme activity during reassociation is more rapid when *D. melanogaster* and *D. virilis* subunits are mixed than when only one kind of subunit is allowed to reassociate. (See MacIntyre [11] for details.) We hope to probe this question further by purifying the enzymes from *D. melanogaster*, *D. simulans*, and *D. virilis* as much as possible and repeating the tests, this time measuring specific activities. Immunological assays may be another way to measure the amount of acid phosphatase protein formed during reassociation.

If, in fact, the technique measures variation in subunit affinities, then what kind and how many amino acid substitutions are responsible for the differences? Obviously, only when amino acid sequences are determined for the acid phosphatases will this question be answered. One could, however, it seems to me, study the relationships with hemoglobin relatively easily. Besides affording an empirical basis for evolutionary comparisons which use the technique of subunit hybridization, such a comparison should also provide useful information to the protein chemist interested in quarternary structure.

There is still another explanation for the results of the interspecific tests which can be ruled out by a closer examination of the data. It might be argued that the degree of the difference between observed and expected homospecific: heterospecific enzyme ratios depends only upon the net charge of the dissociated subunits. That is, if the difference in net charge between the subunit from spe-

cies X and species Y is substantial, then more heterospecific enzymes will form because of the greater electrostatic attraction between the unlike subunits. If this were strictly true, however, one should never see a homospecific:heterospecific enzyme ratio greater than the one expected if subunit association were random. Furthermore, as shown in Figure 12, when the differences between

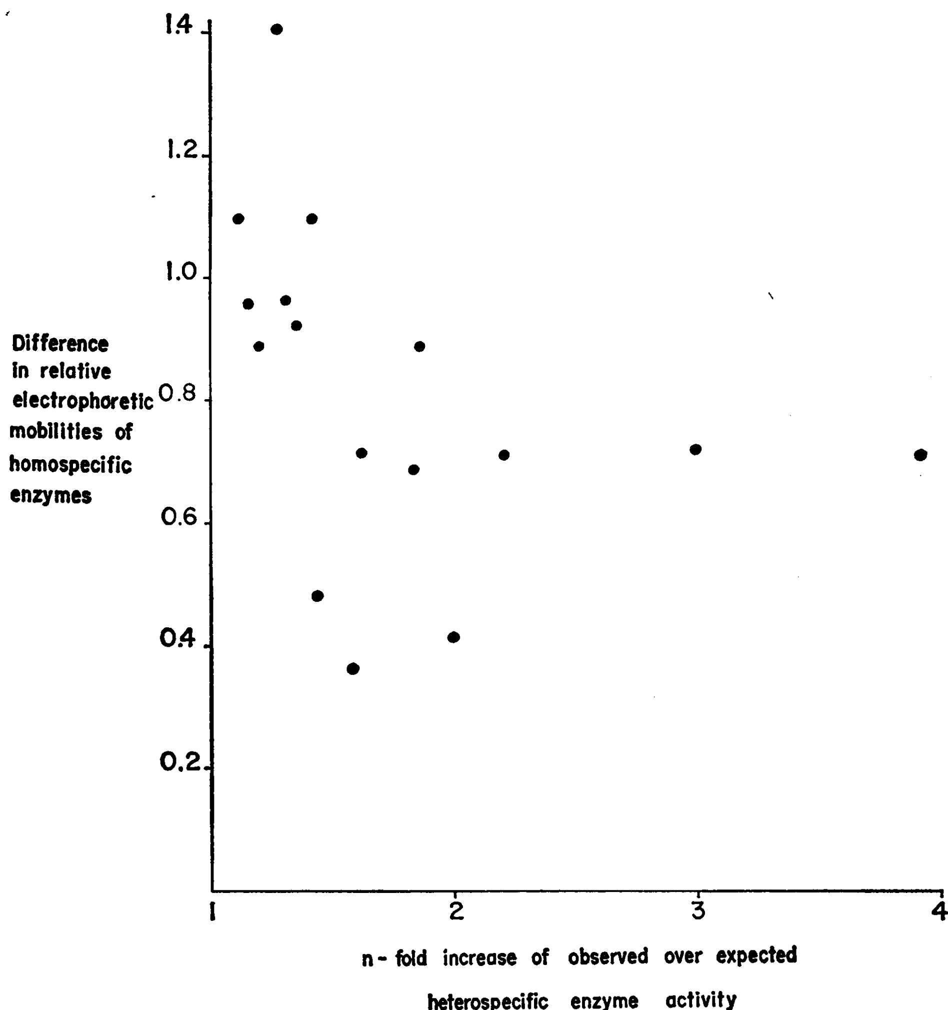


FIGURE 12

Plot of subunit affinity as measured by n-fold increase of observed over expected heterospecific enzyme activity against the difference between the electrophoretic mobilities of the participating homospecific enzymes. Data were taken only from those tests in which the difference between observed and expected homospecific:heterospecific enzyme activities had a minus sign.

observed and expected homospecific:heterospecific enzyme ratios (from the tests in which the heterospecific enzyme activity is greater than expected) are plotted against the distance between the two homospecific enzymes after electrophoretic separation, no definite correlation can be seen.

In order to make Figure 12 understandable, I will discuss how one of the points was plotted. In the *D. melanogaster* × *D. virilis* test, the difference between the two ratios is about 0.7 when the enzyme activities are determined by densitometry. Thus, the actual increase of observed over expected heterospecific enzyme activity in this test is about 3.8. This is plotted on the ordinate. The relative electrophoretic mobilities of the two homospecific enzymes are 1.00 (*D. melanogaster*) and 0.28 (*D. virilis*). The difference of 0.72 is plotted on the abscissa. The point plotted from the coordinates is then determined, and in Figure 12, is the point at the far right on the graph. All the other points representing the other tests in which excessive heterospecific enzyme activity was measured were determined in a similar fashion. Note that if the excess heterospecific enzyme activity were due only to the electrostatic attraction between unlike subunits, the data should plot as a straight line originating at zero.

Despite certain unanswered questions, the technique appears to be quite sensitive in its ability to detect differences between homologous enzymes, even those from sibling species. We were encouraged enough by this conclusion to begin a rather extensive survey of natural populations of *D. melanogaster*, *D. simulans*, and *D. nebulosa*. We hope to detect differences in electrophoretically identical allozymes from different populations, that is, to see if we can extend our present estimates of gene-enzyme variability in natural populations. Also, we hope to determine if the subunits of polymorphic electrophoretic variants of acid phosphatase differ significantly in their ability to form homo- and heterodimeric enzymes. If this could be shown, then it would suggest that the mutational differences between the alleles are not selectively neutral.

7. Summary

Let me conclude with a purposefully overstated summary. I have outlined a technique that allows us to detect differences in the homologous enzymes of closely related species and perhaps even in the same enzyme from different populations within a species. These differences are due to amino acid substitutions affecting subunit association and/or the activity of the enzyme. It is unlikely that either kind of difference would be selectively neutral.

REFERENCES

[1] J. Bell and R. MacIntyre, "An analysis of induced null mutants of acid phosphatase-1 in *Drosophila melanogaster*," *Isozyme Bull.*, Vol. 4 (1971), p. 19.

[2] R. A. Cook and D. E. Koshland, "Specificity in the assembly of multisubunit proteins," *Proc. Nat. Acad. Sci. U.S.A.*, Vol. 64 (1969), pp. 247–252.

[3] E. H. DAVIDSON and B. R. HOUGH, "Genetic information in oocyte RNA," *J. Mol. Biol.*, Vol. 56 (1971), pp. 491–506.

[4] J. FRANKLIN and R. C. LEWONTIN, "Is the gene the unit of selection?," *Genetics*, Vol. 65 (1970), pp. 707–734.

[5] J. E. HABER and D. KOSHLAND, "Relation of protein subunit interactions to the molecular species observed during cooperative binding of ligands," *Proc. Nat. Acad. Sci. U.S.A.*, Vol. 58 (1967), pp. 2087–2092.

[6] N. IWATZUKI and R. OKAZAKI, "Mechanism of regulation of deoxythymidine kinase of *E. coli*. I. Effect of regulatory deoxynucleotides on the state of aggregation of the enzyme," *J. Mol. Biol.*, Vol. 29 (1967), pp. 139–156.

[7] A. KENYON, "Comparison of frequency distributions of viabilities of second with fourth chromosomes from caged *Drosophila melanogaster*, "*Genetics*, Vol. 55 (1967), pp. 123–130.

[8] J. Klotz and D. W. DARNALL, "Subunit structured proteins; A table," *Science*, Vol. 166 (1969), pp. 126–128.

[9] R. MACINTYRE, "Evolution of Acid phosphatase-1 in the genus Drosophila as estimated by subunit hybridization. I. Methodology," *Genetics*, Vol. 68 (1971), pp. 483–508.

[10] ———, "A method for measuring activities of acid phosphatases separated by acrylamide gel electrophoresis," *Biochem. Genet.*, Vol. 5 (1971), pp. 45–56.

[11] ———, "Multiple alleles and gene divergence in natural populations," *Brookhaven Symp. Biol.*, Vol. 23 (1971), in press.

[12] R. MACINTYRE and T. R. F. WRIGHT, "Responses of Esterase-6 alleles of *Drosophila melanogaster* and *D. simulans* to selection in experimental populations," *Genetics*, Vol. 53 (1966), pp. 371–387.

[13] R. W. NOBLE, "Relation between allosteric effects and changes in the energy of bonding between subunits," *J. Mol. Biol.*, Vol. 39 (1969), pp. 479–489.

[14] S. J. O'BRIEN and R. J. MACINTYRE, "Transient linkage disequilibrium in Drosophila," *Nature*, Vol. 230 (1971), pp. 335–336.

[15] M. F. PERUTZ, H. MIURHEAD, J. M. COX, and L. C. G. GOAMAN, "Three dimensional Fourier synthesis of horse oxyhemoglobin at 2.8 Å resolution: The atomic model," *Nature*, Vol. 219 (1968), pp. 131–139.

[16] M. F. PERUTZ and H. LEHMANN, "Molecular pathology of human haemoglobin," *Nature*, Vol. 219 (1968), pp. 902–909.

[17] S. PRAKASH, R. C. LEWONTIN, and J. L. HUBBY, "A molecular approach to the study of genic heterozygosity in natural populations. IV. Patterns of genic variation in central, marginal and isolated populations of *Drosophila pseudoobscura*," *Genetics*, Vol. 61 (1969), pp. 841–858.

[18] C. R. SHAW, "The use of genetic variation in the analysis of isozyme structure," *Brookhaven Symp. Biol.*, Vol. 17 (1964), pp. 117–130.

[19] T. YAMAZAKI, "Measurement of fitness at the esterase-5 locus in *Drosophila pseudoobscura*," *Genetics*, Vol. 67 (1971), pp. 579–603.