EVOLUTION OF ACID PHOSPHATASE-1 IN THE GENUS DROSOPHILA AS ESTIMATED BY SUBUNIT HYBRIDIZATION I: METHODOLOGY

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MANY evolutionary changes in macromolecules have been detected in recent years. The most precise information has come from amino acid sequence comparisons of homologous proteins (see NOLAN and MARGOLIASH (1968) for a review). With the exception of hemoglobin where X-ray diffraction studies on its three-dimensional structure are available (PERUTZ and LEHMANN 1968), it is usually impossible to implicate a functional importance to the differences between two homologous proteins. Indeed, there is a lively dispute concerning the relative importance of natural selection in evolution at the molecular level (KING and JUKES 1969; ARNHEIM and TAYLOR 1969; RICHMOND 1970). Other inter-specific comparisons such as electrophoretic mobilities or immunological characteristics of homologous proteins, in the absence of evidence to the contrary (DUKE and GLASSMAN 1968; HUBBY and THROCKMORTON 1968; ARNHEIM, PRAGER and WILSON 1969), may in fact measure properties of the macromolecule that can vary widely without significantly affecting its performance *in vivo*. Experimental proof that an homologous protein from species X is as or less efficient than the protein from species Y within the cells of an individual from species Y will probably not be obtained in the near future.

It is feasible, however, to investigate a property of a protein that directly affects its biological activity. In multimeric enzymes in which the subunits are inactive, the bonding between the subunits is of considerable importance. Not only must the regions of the subunits involved in this interaction be highly specific for subunit recognition inside the cell, but also the number of kinds of intersubunit bonds may be instrumental in maintaining a subunit-multimer equilibrium (GUIDOTTI, KONIGSBERG and CRAIG 1963). This equilibrium may be important in the regulation of the enzyme's activity. Again, the only specific information available is on hemoglobin, and it is evident that intersubunit bonding involves a considerable number of amino acids (PERUTZ *et al.* 1968). Since intersubunit bonding is such a complex property of the macromolecule, it is presumably subject to the action of natural selection.

There have been a number of qualitative studies on the abilities of homologous subunits from different species to form active heterospecific enzymes (HUBBY and NARISE 1967; MARKERT and WHITT 1968). In general, the subunits, even from species in different classes, are still similar enough to join in heterospecific

combinations. This alone would indicate that the integrity of the regions involved in subunit attachment is selectively important. Can differences in these regions be detected? We have approached this question by measuring the relative activities of the heterospecific and homospecific enzymes formed when subunits of an enzyme from two species are allowed to reassociate together.

For this investigation, the gene-enzyme system acid phosphatase-1 in species of the genus *Drosophila* was chosen. This system has several advantages. First, available evidence indicated it is a simple dimer (MACINTYRE 1966), and methods for reversibly dissociating the enzyme into its constituent subunits have been described (MACINTYRE and DEAN 1967). Secondly, as I will show in the next paper of this series, there is considerable interspecific variation in the electrophoretic mobility of this enzyme. The separability of homospecific and heterospecific enzymes in gels is essential for the measurement of their activities since a quantitation system for measuring the activities of acid phosphatases separated in acrylamide gels has been developed (MACINTYRE 1971). Thirdly, the evolutionary relationships of extant species in the genus *Drosophila* have been thoroughly studied from many points of view (PATTERSON and STONE 1952; THROCKMORTON 1962). Even though the genus *Drosophila* is a small taxonomic group, there is evidence that substantial amounts of gene substitution have taken place during its evolution (LAIRD and MCCARTHY 1968). Several excellent studies on protein evolution have already been made on species within this genus (HUBBY and NARISE 1967; DUKE and GLASSMAN 1968; HUBBY and THROCKMORTON 1968).

The rationale behind the experiments is straightforward. Dissociated subunits from the acid phosphatases from species X and species Y are allowed to join and form the two homospecific enzymes XX and YY, and the heterospecific enzyme XY. The relative activities of each can be measured, and the results compared to an expected distribution based on the assumed amount of X and Y in the observed results using the expansion of $(X + Y)^2$. It is the purpose of this paper to examine and justify several basic assumptions underlying the experimental approach. These assumptions are: (1) the methods used will not favor the formation or quantitation of one of the three enzymes in the reassociated mixture; (2) the methods used do not affect the functional integrity of the dissociated subunits; and (3) acid phosphatase-1 is a dimeric enzyme, or at least is separated into half-size molecules by the dissociation treatment.

Homologous acid phosphatase-1 enzymes from three species will be used in the experiments designed to test the assumptions outlined above. The three include the sibling species *D. melanogaster* and *D. simulans* and the more distantly related *D. virilis*.

MATERIALS AND METHODS

Enzyme purification: Acid phosphatase-1 from the three species was partially purified by the procedures described in MACINTYRE and DEAN (1967). Effective separation of the different species' enzymes was accomplished by chromatography on phosphocellulose. Acid and alkali washed phosphocellulose (Whatman p-11) was packed by gravity into 2×25 cm columns and equilibrated with 0.05 M acetate buffer pH 5.0. Enzyme preparations were dialyzed overnight

against 0.005 M acetate buffer pH 5.0, before layering on the column. After adsorption of the enzyme preparation, 2 column volumes of 0.05 M acetate buffer pH 5.0 were applied. Then 10-ml steps of increasing molarity of acetate buffer were run through the column with enough pressure to maintain a flow rate of 1–2 ml/min. 5-ml fractions were collected. Peak fractions as detected by the enzyme assay described below were pooled and concentrated by making the pooled fractions 70% saturated with ammonium sulfate. The suspension was centrifuged ($27,000 \times g$ for 10 min) and the precipitate redissolved in 0.05 M NaCl.

Enzyme activity assays: Both test-tube and gel assays utilized the same substrate, alpha naphthyl acid phosphate. The hydrolysis releases α -naphthol which couples to the diazonium salt, Fast Red TR. The dye complex is water insoluble. The test-tube reaction involves coupling the α -naphthol with Fast Red TR after a certain period of time. The presence of 4% sodium dodecyl sulfate with the added Fast Red TR solubilizes the dye complex. The gel assay employs the staining mixture described in MACINTYRE (1966) with the omission of magnesium and manganese ions. The bands are cut out of the gel and the dye eluted in measured volumes of glacial acetic acid. The amount of the dye complex in both assays is determined from spectrophotometric readings at 540 nm. Complete details and comparisons of both assays can be found in MACINTYRE (1971).

Electrophoretic procedures: These are described in more detail in MACINTYRE (1971). The apparatus for acrylamide gel electrophoresis described by RAYMOND (1964) for 3 mm thick gels was used and a continuous buffer system of 0.1 M Tris-borate pH 9.2 was employed. Specific modifications included a 5% gel, a prerun of 2½ hr without circulation followed by drainage and replacement of the buffer in the lower compartment, insertion of extracts in 10% dextrose, exposure to a 450-volt potential for the run, and soaking of the gel overnight in cold acetate buffer pH 5.0 before staining.

Checks were always made as described by MACINTYRE (1971) to insure that the enzymes were still catalyzing the reaction at maximum rates when the staining of the gel was terminated.

Dissociation and reassociation procedures: Dissociation of acid phosphatase-1 was accomplished by raising the pH of the extract to between 10 and 11 with dilute NaOH. The description of this procedure and the experimental verification of reversible dissociation can be found in MACINTYRE and DEAN (1967). Reassociation was accomplished by dialysis of alkali-inactivated extracts against 0.4 M Tris-maleate buffers of various pH values. Specific details for each experiment will be given in the RESULTS section. Reassociation of subunits subjected to electrophoresis was accomplished by soaking the gel overnight in a 0.4 M Tris-maleate buffer pH 6.0. The gel was then rinsed in 0.05 M acetate buffer pH 5.0, and stained for acid phosphatase activity.

Methods of estimating molecular weights: 5–20% sucrose gradients were prepared according to the method of MARTIN and AMES (1961) in 0.1 M Tris-Cl buffer pH 7.5. Purified extracts of acid phosphatase-1 were dialyzed overnight against dilute buffer. The standards included purified horse hemoglobin (mol. wt. 67,000) and yeast alcohol dehydrogenase (mol. wt. 152,000). The supernatant of a saturated and centrifuged solution of hemoglobin was used. The concentration of yeast alcohol dehydrogenase was 4 mg/ml. 0.10 ml of a mixture of 9 parts acid phosphatase and 1 part standard was layered on the gradient. Centrifugation was carried out for 15 hr at $68,000 \times g$. Fractions of approximately 0.1 ml (7 drops) were collected and assayed for acid phosphatase and either hemoglobin (0.05 ml diluted to 0.15 ml with water and read at 550 nm in microcuvettes) or alcohol dehydrogenase (0.02 ml added to 1.0 ml of 0.1 M sodium phosphate buffer pH 7.3, containing 0.05 ml ethanol and 200 μ g nicotinamide adenine dinucleotide. Change in OD at 340 nm was followed for 6 min). In another experiment, equal amounts of acid phosphatase-1 from *D. melanogaster* and *D. virilis* were mixed, and 0.10 ml was layered on a gradient. Fractions from this were assayed only for acid phosphatase activity. Electrophoretic retardation coefficients were determined according to the method of ZWANN (1967). Electrophoretic procedures were identical to those described above, except that the gel concentration was either 6 or 9%, depending upon the run. Standards used were hemoglobin, yeast alcohol dehydrogenase, and *E. coli* alkaline phosphatase (mol. wt. 80,000). A voltage drop of 30 v/cm was applied 4 hr for the 9% gel and 2 hr for the 6% gel. Staining for acid phosphatase and detection

of acid phosphate-1 subunits are described above. Methods for staining the standards can be found in KOEN and SHAW (1968). Hemoglobin was visible in the unstained gel.

Interspecific hybrids between *D. melanogaster* and *D. simulans* were obtained from reciprocal crosses observing the precautions discussed in MACINTYRE (1966). Single flies were crushed in 0.08 ml of 0.05 M NaCl, 10% dextrose, and the extract was inserted into acrylamide gel pockets. Electrophoresis, staining, and quantitation of the acid phosphatase zones are described above.

RESULTS

The experimental results will be grouped into three sections, each concerned with one of the three assumptions outlined above. Before the interspecific subunit hybridization experiments can be described, certain preliminary information about the enzymes and the methods of dissociation and reassociation will be presented. This information is relevant to the first assumption and will be presented under that section.

Assumption no. 1: *The methods used do not favor the formation or quantitation of one of the three enzymes in the reassociated mixture.*

A) Preliminary information

1) pH optima of the species' enzymes: If the two homospecific enzymes XX and YY had different pH optima, the measurements of acid phosphatase activity of the three enzymes after electrophoresis and staining would be biased in favor of the enzyme whose pH optimum was closer to the pH of the staining solution. Figure 1 shows that for the three species used in these preliminary experiments, differences in the pH optima of the homologous acid phosphatase-1 enzymes do not exist. Therefore, by incubating the gel in stain in pH 5.0–5.1, there should be no accentuation of the activity of one of the three enzymes. This assumes, however, that the heterospecific enzyme XY will have the same pH optimum as the two homospecific acid phosphatases.

2) Effect of dissociation on acid phosphatase-1 subunits: The pH at which 100% inactivation occurs was determined for each of the three homologous enzymes. The inactivation curves are shown in Figure 2. The experiments have been repeated several times, and the slight differences between the inactivation curves are always obtained. It is thought that dissociation results from the negative intermolecular repulsive forces which build up as the increase in OH⁻ concentration titrates the positively charged polar amino acids. The increase in the net negative charge on the subunit surfaces finally breaks the weak, noncovalent intersubunit bonds (SCHACHMAN 1963). Separated subunits, however, can be further affected by greater increases in pH. The data in Table 1 show that if the pH is raised too high during the dissociation treatment the activity regained after reassociation can be drastically reduced. This may be due to either alterations in the structures of all the subunits or a reduction in the population of subunits that can still reassociate. For this reason, when making an interspecific test it is important to dissociate the acid phosphatase from each species separately to make the treatment as gentle as possible. For example, if one were testing the relative abilities of *D. melanogaster* and *D. virilis* subunits to reassociate and form a heterospecific enzyme, the two enzymes could be mixed and the pH raised to

FIGURE 1.—pH optima of acid phosphatase-1 from *D. melanogaster*, *D. simulans*, and *D. virilis*. Acetate buffers and Tris-maleate buffers were made at the indicated pH. The acetate buffers were all at an ionic strength of 0.1. The ionic strength of the Tris-maleate buffers was not constant. 0.2 ml of partially purified acid phosphatase-1 from each of the three species was incubated for 30 min at 37°C in 2.0 ml of each buffer containing 0.005 M sodium alpha naphthyl acid phosphate. The enzymatic reaction was stopped with the addition of postcoupling solution, and the amount of the α -naphthol-Fast Red TR complex was determined after 1 hr. See MATERIALS AND METHODS for details.

FIGURE 2.—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 (see MATERIALS AND METHODS). Activities were corrected for the volume of NaOH added. The pH at which 100% inactivation occurs for each species' enzyme is indicated on the top line.

11.00 (to completely dissociate the more resistant *D. virilis* acid phosphatase). Far fewer *D. melanogaster* subunits could effectively reassociate, however. This would bias the results strongly toward the formation or measurement of *D. virilis* homospecific enzyme.

3) pH optima for reassociation: If care is taken not to raise the pH too high during the dissociation process, usually from 20–85% of the original activity is regained if the inactivated preparations are dialyzed back against Tris-maleate buffers of high molarity. As Figure 3 indicates, however, the pH optimum for reassociation of the enzyme varies slightly for each species. These differences could be critical in interspecific tests. If the mixture of subunits is dialyzed against a buffer at a pH favoring the reassociation of one homospecific enzyme, the result will be biased. In the initial pairwise tests between the three species which will be described below, reassociation was carried out at each species' pH optimum. If biases in the ratio occur because reassociation takes place under

TABLE 1

Effect of the final pH of the dissociation treatment on the percent of the initial activity regained after reassociation

Final pH of dissociation*	Percent initial activity regained after reassociation
<i>D. melanogaster</i>	
10.79	44.1
11.03	16.2
11.16	9.0
11.44	6.4
<i>D. virilis</i>	
11.00	65.3
11.15	35.9
11.37	14.0
11.55	8.1

* Aliquots of partially purified acid phosphatase-1 preparations from *D. virilis* and *D. melanogaster* were taken to the indicated pH's by addition of 0.1 N NaOH. The aliquots were then assayed to confirm complete inactivation, and dialyzed for 72 hr against 0.4 M Tris-maleate buffer, pH 6.0 at 4°C. They were then assayed for acid phosphatase activity as indicated in MATERIALS AND METHODS.

conditions favoring one or the other of the three enzymes in the mixture, they should be evident from the comparison of the two sets of data.

4) Length of time of reassociation: It is important to allow sufficient time for all undamaged subunits to reassociate in the interspecific tests. Other experiments have indicated that the initial rate of increase in enzyme activity is directly dependent upon the concentration of subunits and the temperature of the Tris-maleate buffer. The final levels of reassociation attained are independent of subunit concentration if dialysis is carried out long enough (MACINTYRE, unpublished). Figure 4 shows that at 4°C, reassociation is virtually complete after 48 hr. In the trial experiments involving reassociation of subunits from *D. melanogaster*, *D. simulans*, and *D. virilis*, the pH-inactivated enzymes were dialyzed for 72 hr at 4°C. This length of time should have assured complete reassociation.

B) Results from interspecific tests

1) Initial tests: Other information relevant to the first assumption depends upon the results of experiments in which dissociated subunits reassociate and form heterospecific enzymes. Three pairwise tests were made; *D. melanogaster* × *D. virilis*, *D. simulans* × *D. virilis*, and *D. melanogaster* × *D. simulans*. In each experiment, equal amounts of a partially purified preparation of each species' acid phosphatase-1 were adjusted to equal activities. Each was warmed to 25°C and taken to the pH of 100% inactivation by adding 0.1 N NaOH. 0.2 ml was assayed to confirm complete inactivation. The dissociated enzymes were kept in ice baths during the assay that confirmed inactivation. If inactivation was complete in both preparations, aliquots of subunits from each species alone and a 1:1 mixture of the two were dialyzed for 72 hr at 4°C against either of two 0.4 M Tris-maleate buffers. The pH of one of these buffers was at the pH optimum for

FIGURE 3.—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 hr at 4°C. The aliquots were then assayed for acid phosphatase activity. Apparent pH optima for each species are indicated on the abscissae.

reassociation of one of the species. The pH of the other buffer was at the other species' reassociation pH optimum. The reassociated preparations were then dialyzed overnight against 0.05 M NaCl and assayed to estimate the percent reassociation. Aliquots from the dialyzed mixtures of species subunits were then

FIGURE 4.—Time course of reactivation of pH-inactivated extracts of acid phosphatase-1 from *D. melanogaster*. 2.0 ml aliquots of a pH-inactivated (10.7) preparation of acid phosphatase-1 from *D. melanogaster* were dialyzed at 4°C against 2 liters of 0.4 M Tris-maleate buffer, pH 6.0. At the times indicated, an aliquot was removed and assayed for acid phosphatase activity as indicated in MATERIALS AND METHODS. The activity of the 72-hr aliquot was 30% of the activity of the initial undissociated preparation.

subjected to electrophoresis, and the acid phosphatase activities of the separated enzymes were estimated as described in MATERIALS AND METHODS.

Figure 5 is an electropherogram of the acid phosphatases from the three species and mixtures (with homospecific and heterospecific enzymes) from each of the three pairwise tests. The heterospecific enzyme forms in each case, strongly suggesting that the three enzymes are homologous. Quantitative results from the three pairwise tests, which were repeated, are in Tables 2–4. Table 5 summarizes the data including the observed and expected (homospecific:heterospecific) enzyme activity ratios and the differences between them. In Table 2, there is an example of how the expected ratio is calculated. Note that the method assumes the enzyme is a dimer. The sign of the difference between the expected and observed ratios indicates which type of enzyme activity, i.e., homospecific or heterospecific, is disproportionally represented in the mixture. A minus sign means the heterospecific enzyme activity is in excess of expected levels. A plus sign indicates the activities of the homospecific enzymes are greater than ex-

TABLE 2
Results of experiments involving reassociation of acid phosphatase-1 subunits of
D. melanogaster and D. virilis

pH of reassociation	Experiment number	Subunits	Percent reactivation after dialysis	Percent of total acid phosphatase in each zone*			Ratio* (homospecific: heterospecific)	Expected ratio**	Difference
				<i>melanogaster</i> homospecific	heterospecific	<i>virilis</i> homospecific			
5.9	1	<i>melanogaster</i>	31						
		<i>virilis</i>	34	9	70	21	0.43	1.00	—0.57
		mixture	52	(7-11)	(67-76)	(17-22)	(0.32-0.49)	(1.00-1.04)	(-0.51-—0.68)
5.9	2	<i>melanogaster</i>	33						
		<i>virilis</i>	35	12	70	18	0.42	1.00	—0.58
		mixture	29	(11-13)	(68-74)	(15-19)	(0.35-0.47)	(1.00)	(-0.53-—0.65)
6.5	1	<i>melanogaster</i>	22						
		<i>virilis</i>	49	10	67	23	0.50	1.00	—0.50
		mixture	38	(9-12)	(64-69)	(21-25)	(0.45-0.56)	(1.00-1.04)	(-0.44-—0.57)
6.5	2	<i>melanogaster</i>	22						
		<i>virilis</i>	47	9	71	20	0.41	1.00	—0.59
		mixture	30	(8-10)	(70-71)	(19-22)	(0.41-0.43)	(1.00)	(-0.59-—0.61)

* Ranges 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 (*melanogaster* subunits) = mean proportion of *melanogaster* homospecific enzyme + $\frac{1}{2}$ mean proportion of heterospecific enzyme, q (*virilis* subunits) = $1-p$. Expected ratio = $\frac{2pq}{p^2 + q^2}$. For example, in experiment 1 at pH 5.9, $p = \frac{9 + \frac{1}{2} (70)}{100} = 0.44$, $q = 1.00 - 0.44 = 0.56$; expected ratio = $\frac{(0.44)^2 + (0.56)^2}{2 (0.44) (0.56)} = 1.00$.

FIGURE 5.—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.

pected. An observed ratio of 1.5 with an expected ratio of 1.0 means that the activity of each homospecific enzyme is about $1\frac{1}{2}$ times that of the heterospecific enzyme. On the other hand, an observed ratio of 0.5 with an expected ratio of 1.0 means that the heterospecific enzyme activity is twice those of the homospecific enzymes. It should be mentioned here that in the tests involving the more distantly related species *D. virilis* $\times$ *D. melanogaster* and *D. virilis* $\times$ *D. simulans*, the heterospecific subunit combination apparently predominates. The opposite appears to be true in the test between the sibling species *D. melanogaster* and *D. simulans*. A possible reason for this will be discussed below.

There are two observations from the results of the interspecific tests pertinent to the first assumption about the methods. The first is the similarity of the results from the two experiments (Table 5). This means that slight differences in enzyme preparations, final pH of dissociation, electrophoretic conditions, etc. will not significantly affect the final results. It is also evident that the results will not be significantly affected if the pH at which reassociation takes place is closer to one species' pH optimum. As long as the pH of reassociation is at one species' pH optimum or somewhere between the two, the data should not be biased.

2) Tests with enzymes purified from phosphocellulose columns: The ability to further purify acid phosphatase-1 by elution from phosphocellulose columns

TABLE 3
Results of experiments involving reassociation of acid phosphatase-1 subunits of
D. simulans and D. virilis

pH of reassociation	Experi- ment number	Subunits	Percent reactivation after dialysis	Percent of total acid phosphatase in each zone*			Ratio* (homospecific: heterospecific)	Expected ratio**	Difference
				<i>simulans</i> homospecific	heterospecific	<i>virilis</i> homospecific			
6.3	1	<i>simulans</i>	53						
		<i>virilis</i>	57	23	63	14	0.60	1.00	-0.40
		mixture	58	(19-28)	(59-67)	(11-18)	(0.49-0.69)	(1.00-1.08)	(-0.31- -0.51)
6.3	2	<i>simulans</i>	27						
		<i>virilis</i>	62	21	56	23	0.79	1.00	-0.21
		mixture	41	(19-22)	(54-60)	(21-24)	(0.67-0.85)	(1.00)	(-0.15- -0.33)
6.5	1	<i>simulans</i>	55						
		<i>virilis</i>	52	28	56	16	0.79	1.00	-0.21
		mixture	59	(26-30)	(53-58)	(15-19)	(0.72-0.89)	(1.00-1.08)	(-0.11- -0.28)
6.5	2	<i>simulans</i>	56						
		<i>virilis</i>	62	18	57	25	0.75	1.00	-0.25
		mixture	70	(17-20)	(53-60)	(23-27)	(0.67-0.89)	(1.00)	(-0.11- -0.33)

* Range in parentheses. Number of determinations: experiment 1 (pH 6.3)-7, (pH 6.5)-7; experiment 2 (pH 6.3)-4, (pH 6.5)-4.

** See footnote of Table 2 for method of calculation.

TABLE 4
Results of experiments involving reassociation of acid phosphatase-1 subunits of
D. simulans and D. melanogaster

pH of reassociation	Experi- ment number	Subunits	Percent reactivation after dialysis	Percent of total acid phosphatase in each zone*			Ratio* (homospecific; heterospecific)	Expected ratio**	Difference
				simulans homospecific	heterospecific	melanogaster homospecific			
5.9	1	simulans	53						
		melanogaster	47	34	41	25	1.44	1.00	+0.44
		mixture	60	(31-37)	(38-43)	(21-29)	(1.33-1.63)	(1.00-1.04)	(+0.29-+0.63)
5.9	2	simulans	28						
		melanogaster	36	35	42	23	1.38	1.00	+0.38
		mixture	34	(33-35)	(41-43)	(23-24)	(1.33-1.44)	(1.00)	(+0.33-+0.44)
6.3	1	simulans	58						
		melanogaster	44	41	40	19	1.50	1.08	+0.42
		mixture	61	(37-45)	(39-41)	(15-24)	(1.44-1.56)	(1.00-1.17)	(+0.27-+0.56)
6.3	2	simulans	62						
		melanogaster	27	34	43	23	1.33	1.00	+0.33
		mixture	41	(33-35)	(41-46)	(19-26)	(1.17-1.56)	(1.00)	(+0.17-+0.56)

* Range in parentheses. Number of determinations: experiment 1 (pH 5.9)-6, (pH 6.3)-7; experiment 2 (pH 5.9)-4, (pH 6.3)-4.

** See footnote of Table 2.

TABLE 5

Summary of results from acid phosphatase-1 subunit reassociation experiments involving *D. melanogaster*, *D. simulans*, and *D. virilis*

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

* Results of two experiments for each interspecific test.

provided an opportunity to obtain more supporting evidence for the first assumption. Specifically, the apparent differences in the reassociation observed in the preliminary interspecific tests are assumed to be due entirely to the properties of the subunits. Biases in the results could occur if other molecules in the partially purified preparations had some effect on the reassociation process. If such molecules do exist, another purification step might change the results of the interspecific tests. This assumes, of course, that the additional purification step would separate acid phosphatase-1 and the other hypothetical macromolecule. Acid phosphatase-1 from *D. melanogaster* and *D. virilis* was eluted from phosphocellulose columns and concentrated as described in MATERIALS AND METHODS. The enzyme from *D. virilis* came off the column when the molarity of the acetate buffer used for elution was between 0.30–0.40. The enzyme from *D. melanogaster* eluted in 0.55–0.65 M. acetate buffer. After concentration of the peak fractions, enzyme preparations were then dissociated, mixed, and reassociated as described above. Measurements of the activities of the three enzymes which formed in the mixture of subunits are in Table 6, experiment 1. A comparison of this ratio with the ratios from the *D. melanogaster* × *D. virilis* tests (reported in Table 5) indicates an increase in purification does not significantly affect the reassociation results.

Since the acid phosphatase-1 enzymes from *D. melanogaster* and *D. virilis* eluted from phosphocellulose columns at very different acetate buffer concen-

TABLE 6
Results from dissociation-reassociation experiments with enzymes eluted from phosphocellulose columns

Experiment number	Enzyme	Dissociated at pH	Reassociated at pH	Percent acid phosphatase activity in each zone*				Ratio* (homospecific: heterospecific)	Expected ratio	Difference
				<i>melanogaster</i> homospecific	heterospecific	<i>virilis</i> homospecific	<i>virilis</i> heterospecific			
1	<i>melanogaster virilis</i>	10.85	6.0	12	67	21	(18-24)	0.49	1.00	-0.51
		11.05		(10-14)	(64-68)	(18-24)		(0.47-0.56)	(1.00-1.04)	(-0.44- -0.53)
2	heterospecific <i>melanogaster-virilis</i>	10.95	6.0	22	67	11	(10-12)	0.49	1.00	-0.51
				(21-23)	(65-69)	(10-12)		(0.45-0.54)	(1.00)	(-0.46- -0.55)
3	heterospecific <i>melanogaster-virilis</i>	10.95	6.0	37	59	4	(3-6)	0.69	1.27	-0.58
				(34-39)	(57-61)	(3-6)		(0.64-0.75)	(1.13-1.27)	(-0.42- -0.63)

* Range in parentheses. Number of determinations: experiment 1-8; experiment 2-3; experiment 3-7.

trations, it was possible to purify the heterospecific enzyme from the *melanogaster* $\times$ *virilis* test. Dissociation and reassociation of this enzyme would be a repeat of the original test, but one in which the initial numbers of subunits from each species should be equal. In the preliminary tests, acid phosphatase activities of the two enzyme preparations were equalized, with the assumption that the numbers of the two kinds of subunits after dissociation would be approximately the same in the mixture. Figure 6 shows that when a reassociated mixture of subunits from *D. melanogaster* and *D. virilis* is eluted from a phosphocellulose column, three peaks of acid phosphatase activity are obtained. If the most active fractions under each peak are pooled, concentrated, and subjected to electrophoresis, the enzymes in each peak can be identified. Figure 7 shows the electropherogram which was obtained. Peak 1 contains the *D. virilis* homospecific enzyme, and peak 3 the *D. melanogaster* homospecific enzyme. The pooled fractions from peak 2 contain only the heterospecific enzyme.

The pH-inactivation curve of this enzyme fell between the curves for *D. melanogaster* and *D. virilis* (MACINTYRE, unpublished). Two preparations of this

FIGURE 6.—Phosphocellulose chromatography profile of acid phosphatase-1 enzymes from re-associated subunits of *D. melanogaster* and *D. virilis*. The column was equilibrated as described in MATERIALS AND METHODS. 20 ml steps of acetate buffer (pH 5.0) of increasing molarity were used for elution. From 0.30 M to 0.40 M, steps were 0.005 M; and from 0.40 M to 0.70 M, steps were 0.02 M. 5 ml fractions were collected and assayed for acid phosphatase activity (see MATERIALS AND METHODS).

FIGURE 7.—Electropherogram of acid phosphatases in peak fractions from the elution profile in Figure 6. Slot 1 contains the three enzymes in the reassociated mixture of *D. melanogaster* and *D. virilis* subunits before adsorption to the column. Slot 2 contains pooled and concentrated fractions from peak 1. Slot 3—pooled and concentrated fractions from peak 2. Slot 4—pooled and concentrated fractions from peak 3.

enzyme were completely dissociated at high pH, and dialyzed back against 0.4 M Tris-maleate buffer pH 6.0. The preparations were then subjected to electrophoresis. Both the homospecific enzymes and the heterospecific enzyme were regenerated, and the measurements of their activities are included in Table 6, experiments 2 and 3. Again, virtually identical differences between observed and expected ratios in this and the earlier tests are obtained. It appears, then, that small differences in the numbers of subunits from the two species will not significantly affect the results of the interspecific tests.

Assumption no. 2: *The methods used do not affect the functional integrity of the dissociated subunits:* The effects on the acid phosphatase-1 enzymes prior to and during the dissociation and reassociation treatments could conceivably alter their structure resulting in distorted (homospecific:heterospecific) enzyme ratios *in vitro*. This can be checked by examining the ratios of the three enzymes in interspecific hybrid flies. It is possible to obtain interspecific hybrids only between the sibling species *D. melanogaster* and *D. simulans*. Another comparison between enzyme ratios obtained *in vivo* and *in vitro* is available, however. This test is between subunits from the two electrophoretic variants of the enzyme in *D. melanogaster*. These are the slow or AA and fast or BB forms of the enzyme

TABLE 7
Acid phosphatase activities of enzymes from D. melanogaster $\times$ *D. simulans interspecific hybrids* and *D. melanogaster* Acph-1^A/Acph-1^B *heterozygotes*
 Also included are the results from reassociated mixtures of subunits from purified and dissociated AA and BB forms of the enzyme from *D. melanogaster*

Test	Percent acid phosphatase activity in each zone*				Ratio* (homospecific: heterospecific)	Expected ratio**	Difference
	<i>melanogaster</i> homospecific	heterospecific	<i>simulans</i> homospecific	homospecific			
<i>D. simulans</i> ♀ × <i>D. melanogaster</i> ♂	21 (17-26)	44 (42-46)	35 (32-40)	1.27 (1.17-1.38)	1.04 (1.00-1.08)	+0.23 (+0.13-+0.38)	
<i>D. melanogaster</i> ♀ × <i>D. simulans</i> ♂	22 (20-25)	43 (40-46)	35 (32-38)	1.33 (1.17-1.50)	1.00 (1.00-1.08)	+0.33 (+0.13-+0.50)	
<i>D. melanogaster</i> <i>Acph-1^A/Acph-1^B</i> heterozygotes	AA 22 (15-26)	AB 45 (41-50)	BB 33 (29-39)	AA+BB:AB 1.22 (1.00-1.44)	1.00 (1.00-1.13)	+0.22 (0-+0.44)	
Dissociated and reassociated AA and BB enzymes from <i>D. melanogaster</i>	15 (10-21)	42 (39-46)	43 (38-48)	1.36 (1.17-1.56)	1.17 (1.13-1.33)	+0.19 (+0.05-+0.36)	

* Range in parentheses. Number of flies analyzed: interspecific hybrids, *D. simulans* parent-8, *D. melanogaster* parent-13; Acph-1^A/Acph-1^B heterozygotes-7. Number of determinations of reassociated AA and BB-7.
 ** See footnote of Table 2.

which are controlled by the *Acph-1^A* and *Acph-1^B* alleles, respectively (MACINTYRE 1966). The electrophoretic patterns of both reciprocal interspecific hybrids between *D. melanogaster* (*Acph-1^B* homozygotes) and *D. simulans* and *Acph-1^A/Acph-1^B* heterozygotes of *D. melanogaster* were assayed. Data are presented in Table 7. Results from the interspecific hybrids can be compared with the ratios reported in Tables 4 and 5.

The data from the adult *Acph-1^A/Acph-1^B* heterozygotes are compared with the patterns in a reassociated mixture of subunits from partially purified and dissociated AA and BB enzymes from *D. melanogaster*. The similarities between *in vivo* and *in vitro* results in both comparisons make it quite probable that the subunits combine in the same proportions or the enzymes which form have the same activities both in the *in vitro* tests and inside living cells. Presumably, therefore, the purification procedures and experimental methods do not significantly alter the functional integrity of the subunits of the enzyme.

Assumption no. 3: *Acid phosphatase-1 is a dimeric enzyme*. Measurements of the molecular weight of the enzyme were made to see if its size is compatible with those of other dimeric enzymes. The acid phosphatases from *D. melanogaster* and *D. virilis* were each centrifuged in sucrose gradients with known standards. The results from the various assays of the collected fractions are depicted in Figure 8. The calculations, following MARTIN and AMES (1961), from the data in A, B, & C of Figure 8 indicate acid phosphatase-1 of each species has a molecular weight of 100,000. The single peak of acid phosphatase activity from the gradient containing a mixture of *D. virilis* and *D. melanogaster* enzymes also indicates the homologous acid phosphatases probably have identical molecular weights.

The ability to detect dissociated subunits in an acrylamide gel following electrophoresis (Figure 9) made it possible to estimate their molecular weight. ZWANN (1967) found that the retardation of the migration of globular proteins in acrylamide gels with smaller pore sizes is inversely related to the log of their molecular weights. Acid phosphatase-1, its subunits, and several known standards were subjected to electrophoresis in 6% and 9% gels. The retardation coefficients have been plotted against the log molecular weight of the standards in Figure 10. The three standards and acid phosphatase-1, whose molecular weights were determined by other methods, fall on the same straight line. The retardation coefficient of the subunits indicates they have a molecular weight of about 55,000. The dissociation treatment, therefore, is splitting acid phosphatase-1 into subunits about half the weight of the native enzyme. This is compatible with a dimer model for active acid phosphatase-1.

DISCUSSION

In the next paper of this series, the results of many experiments involving the formation of heterospecific and homospecific enzymes from subunits of acid phosphatase-1 of different *Drosophila* species will be presented. The interpretation of differences between observed and expected (homospecific:heterospecific) enzyme activity ratios will be that amino acid substitutions have occurred affect-

FIGURE 8.—Sucrose density gradient profiles of acid phosphatase-1 from *D. melanogaster* and *D. virilis*. Gradient A contained acid phosphatase-1 from *D. melanogaster* and purified horse hemoglobin (M.W. 67,000). Gradients B and C contained acid phosphatase-1 from *D. melanogaster* and *D. virilis*, respectively, with yeast alcohol dehydrogenase (M.W. 152,000). Gradient D contained a 1:1 mixture of acid phosphatase-1 from *D. melanogaster* and *D. virilis*.

FIGURE 9.—Electropherogram of acid phosphatase-1 from *D. melanogaster* and *D. virilis* and dissociated subunits of each. Electrophoretic procedures can be found in MATERIALS AND METHODS. The gel buffer, however, was 0.1 M Tris-borate at pH 9.8. The first pocket contains a mixture of acid phosphatase-1 from *D. melanogaster* and *D. virilis*. pH-inactivated enzyme was inserted into the 2nd (*D. melanogaster* and *D. virilis*), 3rd (*D. virilis*), and 4th (*D. melanogaster*) pockets in the gel. Following electrophoresis but before staining, the gel was incubated overnight in 0.4 M Tris-maleate buffer, pH 6.0, to allow the subunits to reassociate.

ing the regions of subunit attachment or the active site itself.

The experiments and the data reported in this paper were designed to test the validity of that interpretation. Before an experiment involving the reassociation of acid phosphatase-1 subunits from two different species is conducted, however, the pH optima, the inactivation curves at high pH, and the pH optima for reassociation should be determined for the enzymes from each species. If this is done, and the proper adjustments are made during the dissociation, reassociation, and gel assay steps in the experiment, there appears to be no reason to suspect bias in the results.

It was shown in three interspecific tests that the results are repeatable, and that they are not affected by further purification of the enzymes or by small differences in the relative proportions of subunits undergoing reassociation. Also, comparisons between the *in vivo* and *in vitro* results from two tests strongly indicate the methods involved in the purification, dissociation, and reassociation of the enzyme do not significantly affect the properties of the subunits necessary for the formation of the active multimeric enzyme.

FIGURE 10.—Retardation coefficients and log of the molecular weights horse hemoglobin (M.W. 67,000), *E. coli* alkaline phosphatase (M.W. 80,000), yeast alcohol dehydrogenase (M.W. 152,000), acid phosphatase-1 and dissociated subunits from *D. melanogaster*. Retardation coefficients are distance (mm) traveled in 9% gel/distance traveled in 6% gel per 1400 volt hours.

The estimates of the molecular weights of the enzyme and its subunits are compatible with a dimeric structure. Many enzymes of similar size are thought, with varying degrees of certainty, to be composed of only two subunits (KLOTZ and DARNALL 1969). Other evidence pointing to a dimeric structure is available. First, an electrophoretic pattern of three bands is not only found in *Acph-1^A/Acph-1^B* heterozygotes and interspecific hybrids but also is obtained after re-association of subunits from different species and after dissociation and reassociation of the purified heterospecific enzyme from the *D. melanogaster* × *D. virilis* test. Such an electrophoretic pattern is considered characteristic of dimeric enzymes (SHAW 1964; MARKERT and WHITT 1968). Secondly, the subunits produced after pH inactivation migrate to a single position in acrylamide gels (Figure 11). Yet they can apparently reassociate and form active enzymes. This observation almost certainly rules out a heteromultimeric structure for acid phosphatase-1, for the subunit controlled by a hypothetical second structural gene would have to have the same net charge and size as the subunit made at the *Acph-1* locus. This conclusion is supported by complementation tests between fifteen ethyl methanesulfonate induced “zero” mutants of acid phosphatase-1. No evidence for a second gene was found (MACINTYRE and J. BELL, unpublished). None of the evidence discussed here, however, conclusively rules out a tetrameric or hexameric arrangement of identical subunits for acid phosphatase-1. End-group analysis and/or peptide fingerprinting of purified enzymes will be necessary to definitely establish the subunit structure of this enzyme.

Even if the subunit which is found after dissociation of the enzyme at high pH

FIGURE 11.—Hypothetical scheme of intersubunit bonds between the homospecific enzymes (A = *D. melanogaster*, B = *D. virilis*) and the heterospecific enzyme (C) formed in the reassociation of *D. melanogaster* and *D. virilis* subunits. (+) and (−) are positively and negatively charged side chains of amino acids. --- indicates weaker hydrogen or hydrophobic bonds between subunits.

is itself composed of more than one polypeptide chain, the calculation of the expected amounts of enzyme which should form during reassociation is still valid. This calculation which follows a binomial expansion assumes only that the treatment splits the enzyme into two identical molecules one-half the size of the native enzyme. The estimate of the molecular weight of the pH-induced subunit of 55,000 makes it quite likely that the native enzyme has been split into halves at high pH.

One final assumption about the methodology of these experiments has not been adequately tested. Calculations of the expected (homospecific:heterospecific) ratio assume that the three kinds of enzymes forming after reassociation have the same specific activities. The differences between observed and expected (homospecific:heterospecific) enzyme activity ratios may result from differences in the affinities of the subunits for one another during reassociation, but alternatively,

the substrate turnover numbers of the various homospecific and heterospecific enzymes may not be the same. It has been impossible to distinguish between these alternative explanations of the results, which themselves are not necessarily mutually exclusive. So far, direct measurements of protein in the zones of acid phosphatase activity have not been possible. The amounts of protein are apparently too small to measure without resorting to radioisotope or immunological techniques. In order to resolve this very serious problem, we are presently trying to directly determine the specific activities of the homospecific and heterospecific enzymes from the three species. Even if we can eventually determine the amount of protein in each zone, however, the results could be misleading since the enzyme has been only partially purified. A contaminating protein migrating to the position of one of the acid phosphatases could lead to a wrong interpretation. Thus we hope to supplement the specific activity determinations with other more indirect experiments. In lieu of completely pure enzyme preparations, however, the issue may never be satisfactorily resolved. Nevertheless, the fact that the observed (homospecific:heterospecific) enzyme activity ratios do not follow the predictions of the binomial expansion suggests that amino acid substitutions affecting either the regions of subunit contact or the conformation of the active site of acid phosphatase-1 have occurred during the evolution of the genus *Drosophila*. Unfortunately, it is impossible to ascertain how many or what kind of amino acid changes these are. It seems unlikely, however, that the amino acid substitutions underlying these differences would be selectively neutral, since they apparently affect such important properties of the enzyme, namely its activity and/or its quaternary structure.

The observation that there may be a preferential formation or an increase in the activity of the heterospecific enzyme in the tests between *D. virilis* and both *D. melanogaster* and *D. simulans* is at first glance rather surprising. The former species is in a different subgenus than *D. melanogaster* and *D. simulans*. One might expect that evolutionary changes in the long time interval separating these species from a common ancestor would have resulted in a decrease in the amount of heterospecific enzyme forming in a mixture of the two kinds of subunits or in its activity. An apparent increase in the expected amount of heterospecific acid phosphatase-1 is seen frequently in other interspecific tests (MACINTYRE, manuscript in preparation).

The substrate specificity of the enzyme from *D. melanogaster* is currently being investigated. It apparently can hydrolyze any aromatic monophosphate but which ones, if any, accumulate in flies homozygous for mutagen-induced "zero" alleles has not yet been determined. Thus at the moment this is only speculation, but there may exist in the cells of individuals of each species an equilibrium between free, inactive subunits and active dimeric enzymes. Such an equilibrium could be important in the regulation of the activity of the enzymes. Thus an excess of intracellular phosphate esters might shift the equilibrium toward the active enzyme, and in the absence of substrates, the inactive subunits might accumulate. In such an equilibrium relationship, the kinds rather than the number of intersubunit bonds would be of primary importance. Therefore, in the test

of subunit reassociation between *D. melanogaster* and *D. virilis* for example, stronger bonds, i.e., ionic rather than hydrophobic, might form in the dimerization of unlike subunits. Figure 11 is a diagrammatic representation of the possible although highly hypothetical interactions. The heterospecific enzyme *in vivo* would be damaging to individuals from either species since it would indiscriminately shift the equilibrium in favor of the active enzyme. At any rate, if the subunit-enzyme equilibrium does exist and is physiologically important, natural selection may not invariably act in such way as to reduce the affinities of subunits of distantly related species.

SUMMARY

A method for detecting what should be selectively important evolutionary changes in a homomultimeric enzyme, acid phosphatase-1, in species of the genus *Drosophila* is proposed. By measuring activity ratios of homospecific and heterospecific enzymes which form after reaggregation of dissociated subunits, differences between amino acid sequences which affect regions of subunit attachment or the conformation of the active site can be detected. Three assumptions about the methodology were examined. First, preliminary information about the homologous enzymes from three species, *D. melanogaster*, *D. simulans*, and *D. virilis* and results from the interspecific subunit reassociation tests indicate the methods used do not bias the results in favor of one or another of the three enzymes which form when subunits from two species reassociate. Secondly, comparisons were made between the results of *in vitro* subunit reassociation experiments and enzyme patterns obtained from both interspecific hybrids and heterozygotes for alleles specifying electrophoretic variants. The similarities between *in vitro* and *in vivo* results make it quite probable that the methods used do not impair the properties of the subunits which are important in reassociation. Thirdly, molecular weight estimates of acid phosphatase-1 and its subunits are compatible with the assumption that the active enzyme is a dimer. Alternative explanations of the results and a hypothetical model of subunit attachment are considered.

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