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Evolution of Acid Phosphatase-1 in the Genus Drosophila. Immunological Studies

Ross J. MacIntyre, Margaret R. Dean, and Gerry Batt

Section of Botany, Genetics and Development, Cornell University, Ithaca, New York, U.S.A.

Summary. The enzyme acid phosphatase-1 was partially purified from 10 Drosophila species. Four antisera were produced and the ten enzymes were reacted against each serum. The method used to quantitate the reactions involved the electrophoretic separation of antigen-antibody complexes from uncomplexed enzyme, followed by densitometry of the free enzyme. Immunological distances were used to obtain correlation coefficients for all pairwise combinations of the 10 species. From these correlation coefficients, a dendrogram was constructed which is very similar to one diagramming the presumed phylogenetic relationships of the ten species. In addition, the data indicate acid phosphatase-1 has evolved at different rates in different lineages within the genus. A preliminary estimate of the unit evolutionary period for this enzyme is 3.25 million years. The method of determining immunological distances which was used in this study is compared to the method of microcomplement fixation in the *Discussion.*

Key words: Drosophila acid phosphatase – Immunological distances – Gel electrophoresis – Antigen:antibody complexes – Unit evolutionary period

Introduction

Immunology has played an important role in systematics and in the study of evolution. When differences between antigens have been used to trace phylogenetic relationships of species and higher taxa, there usually is basic agreement with phylogenies derived from the fossil record (e.g., Goodman, 1963; Mohagheghpoar and Leone, 1969). In fact, phenetic relationships established from the immunological data on a single protein are generally in accord with those determined by taxonomists or comparative morphologists (e.g., Arnon and Neurath, 1969; Lee et al, 1973; Collier and MacIntyre, 1977). This general agreement allows biologists using immunological data to reconstruct a probable course of evolution of related organisms not represented in the fossil record. In addition, antigenic similarities between proteins from the same organism can be used to detect the presence and evolution of duplicated genes (e.g., see Markert et al, 1976; MacIntyre, 1976).

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In some cases, the degree of cross-reaction between antibodies and a heterologous antigenic protein has been calibrated with regard to the amino acid sequence divergence of that protein (Prager and Wilson, 1971; Champion et al, 1975). In these studies, the immunological test employed was microcomplement fixation (Champion et al., 1974). With these calibrated systems, absolute rates of evolutionary change in the protein can be calculated in groups of organisms with well understood phylogenies in which dates for at least some branch points have been independently determined. Very often, such calculations indicate proteins evolve at characteristic and relatively constant rates over time and between lineages. Different rates of change of proteins have been detected in certain lineages within a phylogeny, however. For example, Collier and MacIntyre (1977) showed that α-glycerophosphate dehydrogenase is evolving relatively more rapidly in the Dipteran family Tephritidae, than in the Muscidae and Sarcophagidae.

Although phylogenetic relationships within the genus Drosophila are particularly well understood (Throckmorton, 1975), only two immunological studies have been made on the evolution of specific proteins in this genus (Duke and Glassman, 1968; Collier and MacIntyre, 1977). In both of the cited studies, species relationships, as estimated from the degree of cross-reaction between antisera and homologous proteins (*viz.*, xanthine dehydrogenase or α-glycerophosphate dehydrogenase), are very similar to those established from comparative morphology and/or cytogenetics. In addition, α-glycerophosphate dehydrogenase appears to be evolving very slowly. Only 8 or 9 amino acid differences can be detected between the enzymes from the most distantly related Drosophila species.

In this report, we present the data from an immunological study of another Drosophila enzyme, acid phosphatase-1. This lysosomal gene-enzyme system has been studied in detail in *D. melanogaster* (MacIntyre, 1966; Anastasia-Sawicki and MacIntyre, 1976; Morrison and MacIntyre, 1977). It contrasts markedly with α-glycerophosphate dehydrogenase. First, unlike α-glycerophosphate dehydrogenase (Collier, 1977), there is extensive interspecific variation in its electrophoretic mobility (MacIntyre, 1972) and in its levels of intraspecific polymorphism (see Ayala and Tracey, 1974). Second, and again unlike α-glycerophosphate dehydrogenase (O'Brien et al, 1973), acid phosphatase-1 null mutant homozygotes (Bell et al., 1972) are apparently as viable as their wild type siblings in the laboratory environment (Ogah and MacIntyre, 1974). And finally, studies on the evolution of acid phosphatase-1, which involve in vitro subunit hybridization (MacIntyre, 1971a, and see the accompanying paper) indicate amino acid substitutions have occurred which affect the subunit contact regions of the protein. We present evidence in an accompanying paper that the few amino acid substitutions in Drosophila α-glycerophosphate dehydrogenase do not affect subunit binding (Collier and MacIntyre, 1978).

We will show in this immunological study of acid phosphatase-1 in the genus Drosophila that this protein is evolving considerably more rapidly than α-glycerophosphate dehydrogenase. Also, the species relationships established from these data are generally similar to those in the phylogeny of Throckmorton (1974), even though differences in the rate of amino acid substitution in the enzyme during the evolution of some species groups can be detected.

Materials and Methods

Drosophila Species. The stock of *D. melanogaster,* monomorphic for the common acid phosphatase-1 allozyme (*Acph-1*^{BB}) was collected at Commack, New York in 1964 (see MacIntyre, 1966, for details). Stocks of nine other species were obtained from the University of Texas, Drosophila species collection center. They are (collection numbers are in parentheses): *D. simulans* (H48.3), *D. nebulosa* (2373.9), *D. willistoni* (2268.20), *D. paulistorum* (1957.2), *D. emarginata* (H158.2), *D. immigrans* (2321.9), *D. virilis* (1801.1), *D. mercatorum* (2507.7), and *D. mulleri* (2533.2).

Partial Purification of Acid Phosphatase-1. Enzyme from each of the ten species was partially purified according to MacIntyre (1971a). The enzyme preparations after the last purification step, phosphocellulose chromatography, were approximately thirty-fold purer than crude extracts. All preparations exhibited a single zone of acid phosphatase activity following acrylamide gel electrophoresis and staining.

Electrophoretic and Densitometric Procedures. Acid phosphatase activity was assayed either spectrophotometrically, or densitometrically following polyacrylamade gel electrophoresis of extracts, according to the methods of MacIntyre (1971b). All partially purified preparations used in these studies were diluted with 0.05 m NaCl containing bovine serum albumin at 17 mgm/ml to 27 units of acid phosphatase activity (where 1 unit = Δ $OD_{540m\mu}$ of .075). Aliquots of these diluted and adjusted extracts were stored frozen at -20°C (without loss of acid phosphatase activity) until their use during these experiments.

Polyacrylamide gels were stained for 8 h at 30°C and then kept in 5% acetic acid. Zones of acid phosphatase activity in the gel exhibit linear rates of dye deposition up to 12 h at 30°C (MacIntyre, 1971b). Enzyme activity in these zones after densitometry is expressed as the area under the curve in square millimeters.

Preparation of Antibodies. The exact injection and bleeding procedures followed are outlined by Bell and MacIntyre (1973). Partially purified enzymes from four species, *D. melanogaster, D. nebulosa, D. paulistorum* and *D. virilis* were used as antigens. Single antisera against acid phosphatase from *D. nebulosa, D. paulistorum* and *D. virilis* were used in these experiments. For *D. melanogaster*, however, two antisera were prepared and used in the interspecific tests described below.

Enzyme Inhibition Tests. 0.2 ml aliquots of partially purified enzyme (adjusted to 27 units/0.2 ml) were mixed with 0.2 ml of undiluted pre-immune serum (controls) or 0.2 ml of immune sera at different dilutions (all serum dilutions were in 0.05 m NaCl, 17 mgm/ml bovine serum albumin). The mixtures were incubated at 4°C for 24 h, then centrifuged for 10 minutes in a table top centrifuge (International). After centrifugation, 0.10 ml of the supernatants was mixed with 0.10 ml of 0.05 m NaCl that 40% V/V glycerol and contained the dye marker bromphenol blue at a concentration of 2 mgm/ml. 0.030 ml from each mixture was then inserted into a pocket at the top of a polyacrylamide gel (see above). The presence of the dye allowed us to make sure that all of the sample was inserted correctly into the gel pocket. The gels were subjected

to electrophoresis for either 1800 volt h (tests with enzymes from *D. nebulosa*, *D. paulistorum*, *D. willistoni*, *D. simulans* or *D. melanogaster*) or for 3000 volt h (tests with enzymes from *D. immigrans*, *D. virilis*, *D. mercatorum*, or *D. mulleri*). Following electrophoresis, the gels were soaked overnight in 0.05 M acetate buffer, pH 5.0 at 4°C and then stained for acid phosphatase activity (MacIntyre, 1971b). Calculations of the extent of inhibition and of immunological distances are described in the *Results* section.

Results

Electrophoretic Mobilities of the Species' Enzymes. Figure 1 is an electropherogram of the relative electrophoretic mobilities of the acid phosphatases in partially purified extracts from the 10 species. The evidence that these enzymes are homologous is presented elsewhere (MacIntyre, 1972). The enzymes from the three species from the *willistoni* species group, *viz.*, *D. willistoni*, *D. paulistorum* and *D. nebulosa* have the fastest rate of migration. Conversely the enzymes from *D. virilis*, *D. mercatorum* and *D. mulleri*, three species from the subgenus *Drosophila*, have the slowest and, in fact, identical electrophoretic mobilities under these conditions. The electrophoretic variation exhibited by the acid phosphatases from the ten species is very probably due to

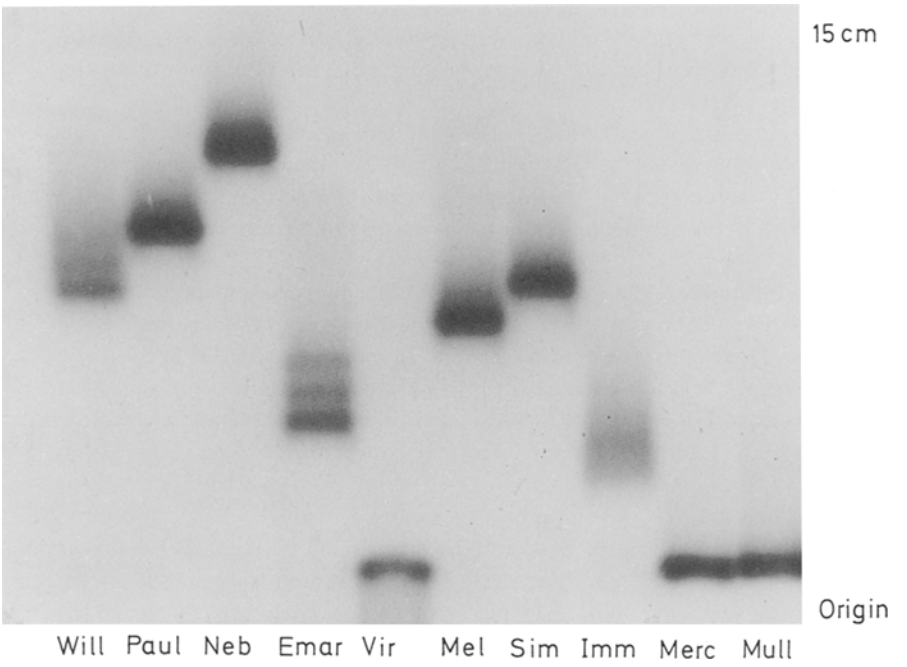

Fig 1. Electropherogram of the acid phosphatases from the 10 Drosophila species in this study. The direction of migration is upward. Species abbreviations (left to right) are as follows: *Will* = *D. willistoni*, *Paul* = *D. paulistorum*, *Neb* = *D. nebulosa*, *Emar* = *D. emarginata*, *Vir* = *D. virilis*, *Mel* = *D. melanogaster*, *Sim* = *D. simulans*, *Imm* = *D. immigrans*, *Merc* = *D. mercatorum*, *Mull* = *D. mulleri*

to net charge differences, since MacIntyre (1971a) has shown that the molecular weights of the homologous enzymes from *D. melanogaster* and *D. virilis* are the same. Subbands can be seen in the enzyme patterns from *D. willistoni, D. emarginata* and *D. immigrans.* All the individual flies from the stocks of these three species used in this study produce subbanded acid phosphatase patterns, ruling out the possibility that the several bands are due to allozyme polymorphism. Presently, we do not know what the molecular basis of this subbanding is. In the tests discussed below, however, each of the bands in a pattern is inhibited to the same extent by any particular antiserum.

Measurement of the Antigen-Antibody Reaction. The strength of the reaction between the homologous or heterologous antigenic acid phosphatase and various dilutions of an immune serum was determined by densitometry of active enzyme after electrophoresis. This was necessary because acid phosphatase-antibody complexes retain enzymatic

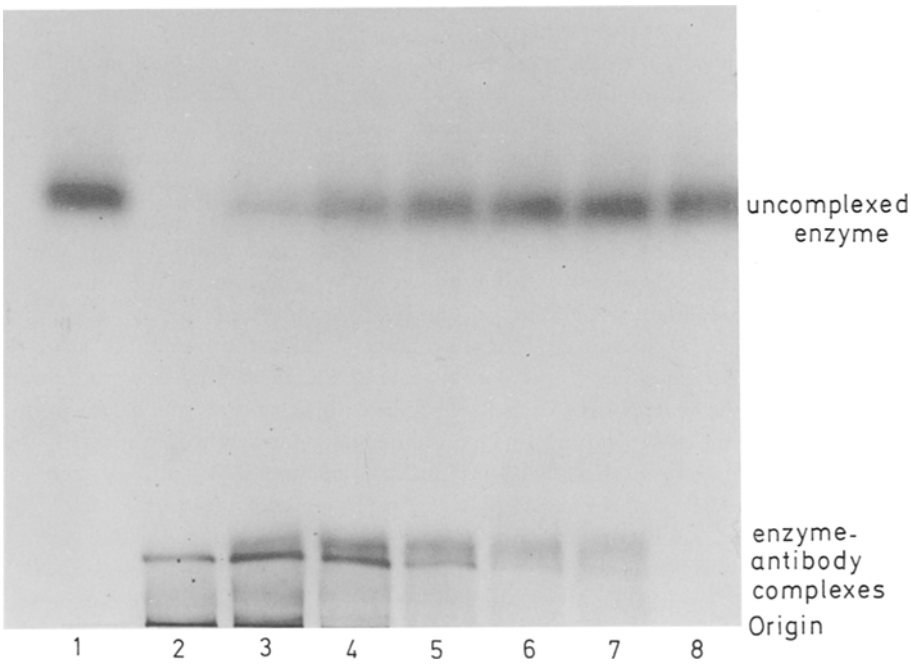

Fig 2. Electropherogram showing the results of an inhibition test. In this gel, acid phosphatase from *D. paulistorum* had been incubated with either pre-immune serum (alleys 1 and 8) or immune serum (against acid phosphatase from *D. nebulosa*) at the following dilutions: 1:10 (alley 2), 1:20 (alley 3), 1:30 (alley 4), 1:40 (alley 5), 1:60 (alley 6), and 1:100 (alley 7). The direction of migration is upward. Note the presence of enzymatically active antigen-antibody complexes near the origin and in the gel pockets. Inhibition is complete at the 1:10 dilution of the antiserum, i.e., no free enzyme activity is evident (alley 2). On the other hand, there is no difference detectable by densitometry between the amount of free enzyme after incubation with immune sera diluted 1:100 or with pre-immune serum (alleys 7 and 8), even though some enzymatically active complexes can be seen near the origin in alley 7.

activity and many remain in solution after centrifugation, particularly if either the antigen or antibody is in excess in the mixture. Soluble, enzymatically active complexes can be cleanly separated from active, uncomplexed enzyme by gel electrophoresis, however (see Tengerdy and Faust, 1971). Thus, the amount of uncomplexed enzyme remaining after the reaction between antigen and antibody can be measured directly. Figure 2 is a photograph of a gel which shows the separation of complexes from uncomplexed enzyme. The enzymatically active complexes remain near the origin of the gel. In this figure, the activity of the acid phosphatase from *D. paulistorum* remaining after incubation with pre-immune serum is shown in alleys 1 and 8. Alleys 2-7 show how much uncomplexed enzyme remains after incubation with increasing dilutions of immune serum (against the enzyme from *D. nebulosa*). At the highest concentration of antibodies, alley 2, all of the enzyme from *D. paulistorum* is complexed. As the antibody concentration decreases, the amount of free enzyme increases. The activity of free enzyme after incubation with a dilution of immune sera divided by the activity of the free enzyme after incubation with pre-immune sera (X100) provides an estimate of the degree of complexing of the enzyme at the particular immune serum dilution. The complexing of acid phosphatase by antibodies will be referred to in the text and in Table and Figure legends as "inhibition". It should be kept in mind that the term inhibition does not imply inhibition of enzyme activity in this system, but rather, the inhibition of free movement of the enzyme in the acrylamide gel. In general, each species' enzyme was tested against each antiserum at several dilutions extending from a dilution which gave total complex formation or inhibition to one which left a level of free enzyme activity not measurably different from that remaining after incubation with pre-immune sera. Each test was performed in duplicate or, in some cases, in triplicate.

We have not included tables containing all the raw data in terms of percent inhibition of each species enzyme at the various dilutions of each of the five antisera. We will make these available upon request, however. In general, each undiluted antiserum reacts at least partially with the enzymes from all ten species. In a few cases, the degree of inhibition or complexing of the enzyme by an undiluted antiserum, however, is very slight, *e.g.*, the enzymes from *D. melanogaster* and *D. simulans* are only slightly complexed by the antibodies against the acid phosphatase from *D. nebulosa* and from *D. virilis*. In addition, antibodies from one of the two rabbits immunized with a partially purified *D. melanogaster* extract fail to form detectable complexes with the acid phosphatase from *D. virilis*. The other rabbit produced antibodies which did complex with the *D. virilis* enzyme, but the cross-reaction was very weak. The differences between the antisera concentrations necessary to equivalently inhibit the enzymes from the various species will be discussed below.

The mean estimates of percent inhibition can be plotted against the antiserum concentrations. As in other such immunological studies (e.g., Tafler et al., 1973), the estimates of percent inhibition tend to fall on a straight line if the axis containing the antiserum dilutions is logarithmic, *i.e.*, percent inhibition is inversely proportional to the log of the antiserum concentration. It is possible to use the raw data from the repeated determinations and calculate regression lines for the tests of each species' enzyme against each antiserum. From these calculated regression lines, the best estimate of the antiserum concentration at which 50% inhibition of the enzyme occurs can be determined. In addition, the standard error of that determination can be used to obtain

the 95% confidence limit surrounding that estimate. These values, which were calculated for each species' enzyme with each antiserum, are presented in Tables 1-5. The exact statistical tests applied are referenced in the footnote to Table 1.

Table 1. Summarized results from tests with the antiserum against the acid phosphatase from *D. nebulosa*

Drosophila Species	Antiserum Dilution at 50% Inhibition*	95% Confidence Limits*	Slope * ± S.E.	Index of Dissimilarity	Minimum and Maximum Indices of Dissimilarity**	Immunological Distance	Minimum and Maximum Immunological Distances**
nebulosa	105	18, 129	-111.5 ± 5.9				
willistoni	39	32, 47	-99.4 ± 6.6	2.69	1.83, 4.03	42	26, 61
paulistorum	35	30, 41	-121.5 ± 6.2	3.00	2.09, 4.30	48	32, 63
emarginata	12	8.6, 16	-84.0 ± 4.9	8.75	5.37, 15.00	94	73, 118
virilis	2.0	1.7, 2.5	-152.8 ± 7.7	52.50	34.40, 75.88	172	154, 188
immigrans	1.9	1.6, 2.3	-93.1 ± 3.5	55.26	37.39, 80.62	174	157, 191
mulleri	1.7	1.3, 2.3	-131.5 ± 8.8	61.76	37.39, 99.23	179	157, 200
mercatorum	1.4	1.2, 1.8	-147.4 ± 9.8	75.00	47.78, 107.50	187	168, 203
melanogaster	0.6	0.5, 0.8	-68.1 ± 3.8	175.00	107.50, 258.00	224	203, 241
simulans	cannot estimate						

*Taken from regression lines of the log of the antiserum concentrations versus percent inhibition estimates. The 95% confidence limits were calculated according to Snedecor and Cochran (1971), pages 159-160.

**See text and Fig. 3 for a description of the minimum and maximum indices of dissimilarity and immunological distances.

Table 2. Summarized results from tests with the antiserum against the acid phosphatase from *D. paulistorum**

Drosophila Species	Antiserum Dilution at 50% Inhibition	95% Confidence Limits	Slope ± s.e.	Index of Dissimilarity	Minimum and Maximum Indices of Dissimilarity	Immunological Distance	Minimum and Maximum Immunological Distances
paulistorum	198	140, 277	-68.4 ± 8.7				
willistoni	115	77, 172	-123.8 ± 19.3	1.72	1, 3.60	24	0, 56
nebulosa	19	16, 24	-127.2 ± 6.9	10.42	5.83, 17.31	102	77, 123
emarginata	9	6.7, 12	-128.6 ± 12.9	22.00	11.67, 41.34	134	107, 162
immigrans	3	1.9, 4.0	-97.1 ± 8.5	66.00	35.00, 145.78	182	154, 216
melanogaster	1.8	0.7, 4.3	-84.4 ± 12.8	110.00	32.55, 395.77	204	151, 260
virilis	1.6	0.9, 2.8	-103.0 ± 13.4	123.75	50.00, 307.78	209	170, 249
mulleri	1.6	1.3, 2.0	-168.1 ± 10.2	123.75	70.00, 213.08	209	185, 233
mercatorum	1.4	1.0, 1.9	-205.9 ± 19.4	141.43	73.68, 277.00	215	187, 244
simulans	1.4	1.1, 1.8	-128.4 ± 9.1	141.43	77.78, 251.82	215	189, 240

*See footnotes for Table 1.

Table 3. Summarized results from tests with the antiserum against the acid phosphatase from *D. virilis**

Drosophila Species	Antiserum Dilution at 50% Inhibition	95% Confidence Limits	Slope ± s.e.	Index of Dissimilarity	Minimum and Maximum Indices of Dissimilarity	Immunological Distance	Minimum and Maximum Immunological Distances
virilis	277	205, 373	-113.1 ± 7.3				
mercatorum	14	12, 17	-120.8 ± 6.4	19.79	12.06, 31.08	130	108, 149
mulleri	5	4.6, 5.9	-167.7 ± 8.2	55.40	34.75, 81.09	174	154, 191
willistoni	3.0	2.2, 4.1	-90.7 ± 5.3	92.33	50.00, 169.55	197	170, 223
immigrans	2.7	2.1, 3.4	-97.4 ± 4.4	102.59	60.29, 177.62	201	178, 225
nebulosa	2.7	2.1, 3.0	-88.8 ± 5.2	102.59	68.33, 177.62	201	183, 225
emarginata	2.0	1.6, 2.4	-81.3 ± 3.6	138.50	85.42, 233.12	214	193, 237
paulistorum	1.7	1.4, 2.1	-111.0 ± 5.7	162.94	97.61, 266.43	221	199, 243
melanogaster	cannot estimate						
simulans	cannot estimate						

*See footnotes for Table 1.

Table 4. Summarized results from tests with the antiserum from rabbit No. 1 against the acid phosphatase from *D. melanogaster**

Drosophila Species	Antiserum Dilution at 50% Inhibition	95% Confidence Limits	Slope ± s.e.	Index of Dissimilarity	Minimum and Maximum Indices of Dissimilarity	Immunological Distance	Minimum and Maximum Immunological Distances
melanogaster	1509	1037, 2204	-83.7 ± 5.7				
simulans	1236	925, 1663	-97.8 ± 5.3	1.22	1.00, 2.38	9	0, 38
immigrans	88	62, 124	-74.0 ± 5.2	17.15	8.36, 35.55	123	92, 155
paulistorum	80	65, 99	-111.2 ± 5.5	18.86	10.47, 33.90	128	102, 153
nebulosa	71	57, 88	-98.0 ± 5.1	21.25	11.78, 38.67	133	107, 159
willistoni	62	47, 80	-108.2 ± 8.1	24.33	12.96, 48.69	139	111, 167
emarginata	48	34, 66	-80.4 ± 5.3	31.43	15.71, 64.82	150	120, 181
mulleri	10	7.7, 14	-119.8 ± 8.4	150.90	74.07, 286.23	218	187, 246
mercatorum	9	7.0, 11	-124.5 ± 5.9	167.67	94.27, 314.86	222	197, 250
virilis	2.2	1.6, 3.1	-136.7 +12.1	685.90	334.52, 1377.50	284	252, 314

*See footnotes for Table 1.

Table 5. Summarized results from tests with the antiserum from rabbit No. 2 against the acid phosphatase from *D. melanogaster**

Drosophila Species	Antiserum Dilution at 50% Inhibition	95% Confidence Limits	Slope ± s.e.	Index of Dissimilarity	Minimum and Maximum Indices of Dissimilarity	Immunological Distance	Minimum and Maximum Immunological Distances
melanogaster	620	449, 855	-77.3 ± 5.0				
simulans	545	364, 816	-87.0 ± 7.3	1.14	1.00, 2.35	6	0, 37
willistoni	19	14, 25	-114.3 ± 9.5	32.63	17.96, 61.07	151	125, 179
paulistorum	19	14, 27	-92.6 ± 7.7	32.63	16.62, 61.07	151	122, 179
nebulosa	17	14, 21	-92.1 ± 4.9	36.47	21.38, 61.07	156	133, 179
emarginata	7.0	6.1, 8.8	-131.2 ± 6.2	88.57	51.02, 140.16	195	171, 215
immigrans	7.0	5.1, 9.9	-111.8 ±10.3	88.57	45.35, 167.65	195	166, 222
mulleri	2.0	1.6, 2.5	-128.2 ± 7.5	310.00	179.60, 534.37	249	225, 273
mercatorum	1.3	1.2, 1.6	-131.3 ± 5.2	476.92	280.62, 712.50	268	245, 285
virilis	cannot estimate						

*See footnotes for Table 1.

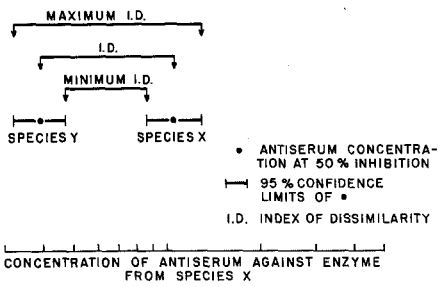

Fig. 3. Diagram of the relationships between minimum and maximum indices of dissimilarity

Interspecific Comparisons. The antiserum concentration producing 50% inhibition of the homologous enzyme, i.e., the enzyme used to elicit the antibodies, is the basic datum for all subsequent interspecific or heterologous comparisons. In order to express the degree of cross-reaction between a heterologous antigen and an antiserum, a so-called "index of dissimilarity" is generally used, from which an "immunological distance" is then determined. In our study, the index of dissimilarity is the factor by which the antiserum must be concentrated from that dilution producing 50% inhibition of the homologous enzyme (the homologous value) in order to inhibit 50% of the activity of a heterologous enzyme (the heterologous value). Again, the upper and lower 95% confidence limits of each index of dissimilarity can be determined. Figure 3 diagrams how these upper and lower limits, or what we call the "maximum" and "minimum" indices of dissimilarity, were determined in this study. The latter is the n-fold difference between the antiserum concentrations representing the upper 95% confidence limit of the heterologous value and the lower 95% confidence limit of the homologous value. Conversely, the maximum index of dissimilarity is the n-fold difference between antiserum concentrations representing the lower 95% confidence limit of the heterologous value and the upper 95% confidence limit of the homologous value. Finally, the mean, minimum and maximum indices of dissimilarity can be converted to mean, minimum and maximum "immunological distances" by multiplying the $\log_{10}$ of each index by 100. This allows one to visualize the differences in the extent of cross-reaction of the enzymes from a number of species on a linear scale.

The mean, minimum and maximum indices of dissimilarity and immunological distances for the tests with each of the five antisera are contained in Tables 1-5. In addition, Figures 4-7 show the relationships between the acid phosphatases of the Drosophila species used in this study in terms of immunological distances. Clearly, there are interspecific differences in the structure of acid phosphatase which can be detected immunologically. Indeed, the comparisons indicate that in each antiserum test groups of species can be defined with regard to statistically significant immunological distance differences. Thus, in the tests with antibodies against the enzyme from *D. nebulosa* (see Fig. 4), the group with an acid phosphatase most similar to that from *D. nebulosa* contains *D. paulistorum* and *D. willistoni*. *D. emarginata* is the sole representative of a second "group", whereas the species from the subgenus Drosophila, *viz.*, *D. virilis*, *D. mercatorum*, *D. mulleri* and *D. immigrans* comprise a third group. Finally, the last group, *i.e.*, one whose acid phosphatases only very weakly react with the anti-*D. nebulosa* serum, includes the subgenus Sophophora species, *D. melanogaster* and

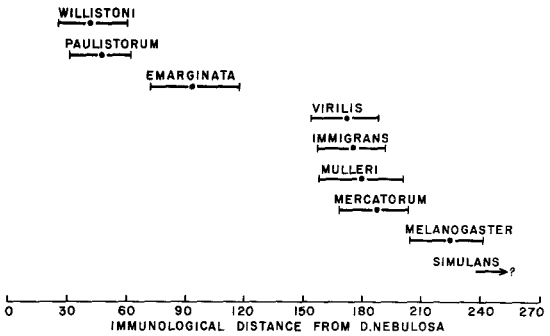

Fig. 4. Graph of the immunological distances between the acid phosphatases of the Drosophila species used in this study, as determined from the antiserum against the enzyme from *D. nebulosa*. • = mean immunological distance. ⊢⊣ = minimum and maximum immunological distances. → ? = cross-reaction was too weak to provide a reliable estimate of immunological distance

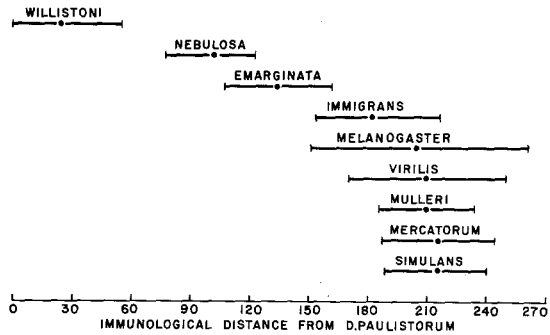

Fig. 5. Graph of the immunological distances between the acid phosphatases of the Drosophila species used in this study, as determined from the antiserum against the enzyme from *D. paulistorum*. • = mean immunological distance. ⊢⊣ = minimum and maximum immunological distances

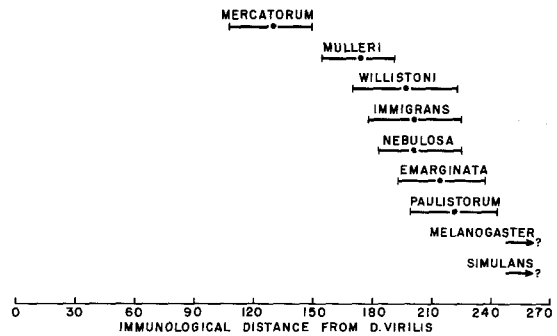

Fig. 6. Graph of the immunological distances between the acid phosphatases of the Drosophila species used in this study, as determined from the antiserum against the enzyme from *D. virilis*. • = mean immunological distance. ⊢⊣ = minimum and maximum immunological distances. → ? = cross-reaction was too weak to provide a reliable estimate of immunological distance

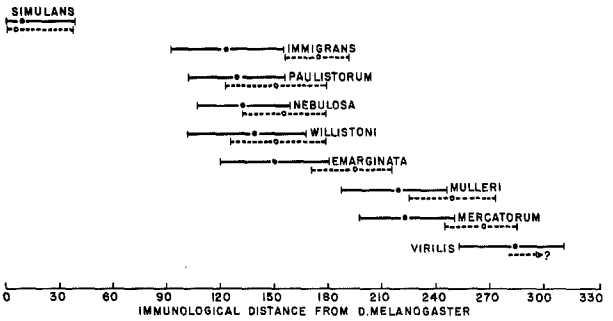

Fig. 7. Graph of the immunological distances between the acid phosphatases of the Drosophila species used in this study, as determined from the two antisera against the enzyme from *D. melanogaster*. • = mean immunological distance. ⟝⟞ = minimum and maximum immunological distances. ⟶ ? = there was no detectable cross reaction between the antiserum from rabbit no. 2 and the enzyme from *D. virilis*. —— = results from tests with the antiserum from rabbit no. 1. —— = results from tests with the antiserum from rabbit no. 2

D. simulans. In tests with antibodies against the acid phosphatase from *D. paulistorum* (Fig. 5), the groups are not quite as clearly defined. The difference between the enzymes from *D. willistoni* and *D. paulistorum* in tests with this antiserum is not significant. *D. nebulosa* and *D. emarginata* form a second fairly closely related group while the remaining species fall into a third relatively homogenous group in terms of their immunological distances from *D. paulistorum*. The antibodies against the acid phosphatase from *D. virilis* do not strongly cross-react with the enzymes from any of the nine species. The immunological distance from *D. virilis* to *D. mercatorum* is significantly shorter than those from *D. virilis* to any of the other species. Of the species falling into a second group, *D. mulleri* is the species most closely related to *D. virilis* both phylogenetically (see below) and in terms of the immunological distances shown in Figure 6. Again, the acid phosphatases from *D. melanogaster* and *D. simulans* only barely complex with the antibodies in the undiluted antiserum against the enzyme from *D. virilis*. The results are qualitatively the same in the two series of tests involving antisera against the acid phosphatase from *D. melanogaster* (see Fig. 7). *D. melanogaster's* sibling species, *D. simulans*, has an enzyme which reacts essentially as well with the anti-*melanogaster* sera as does the homologous antigen. In terms of increasing immunological distances from *D. melanogaster*, three other distinct groups are evident, a large group containing *D. immigrans*, *D. paulistorum*, *D. nebulosa*, *D. willistoni* and *D. emarginata*, a group containing *D. mercatorum* and *D. mulleri*, and finally and least closely related to *D. melanogaster*, a monospecific group containing *D. virilis*. Indeed there is no cross-reaction between *D. virilis's* acid phosphatase and the antibodies made by one of the rabbits against the same enzyme from *D. melanogaster*.

Species Relationships. The relationships of the species, as defined by these immunological distances, can be visualized separately for each tested antiserum in Figures 4-7. The whole data set, however, can be used to construct a more meaningful dendrogram. In order to do this, we calculated the correlation coefficients for each pair of species using as x and y the logarithms of the antiserum concentrations necessary to inhibit

or complex 50% of their respective acid phosphatase activities. For example, in comparing *D. simulans* and *D. virilis*, the following data were analyzed:

Antiserum against	*A**	*LogA*	*B**	*LogB*
D. nebulosa	<1	0	2.0	0.30
D. virilis	<1	0	2.77	2.44
D. paulistorum	1.4	0.15	1.6	0.20
D. melanogaster (rabbit no. 1)	1236	3.09	2.2	0.34
D. melanogaster (rabbit no. 2)	545	2.74	<1	0

Slope = -0.2836
Y intercept = 0.9985
σLogX = 1.4090
σLogY = 0.8997
r = -0.4484

*Where A equals the antiserum dilution necessary to inhibit 50% of the enzyme activity from *D. simulans* and B equals the antiserum concentration necessary to inhibit 50% of the enzyme activity from *D. virilis*.

This procedure is adapted from that of Lee (1968). The complete matrix of r values is presented in Table 6. Closely related pairs of species should have a high, positive correlation coefficient, whereas more distantly related species will either show a lack of correlation, or possibly a negative correlation if the enzymes from the two species alternate in exhibiting strong and weak cross-reactions against the several antisera.

A dendrogram, in which the species are clustered according to the matrix of r values, is shown in Figure 8. Construction of the dendrogram proceeded according to the rules outlined by Sneath and Sokal (1973) for the unweighted pair group method. Clustering was attempted at successive intervals of 0.10, beginning at r = +1.00 to +0.91 and proceeding downward. In these immunological tests, similarities in the responses of the acid phosphatases from the sibling species pairs, *D. melanogaster* and *D. simulans*, on the one hand, and *D. willistoni* and *D. paulistorum* on the other, resulted in their having correlation coefficients greater than +0.99. These pairs, then, formed the first clusters

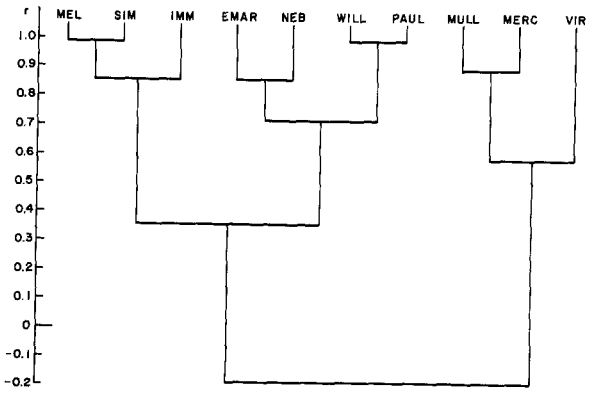

Fig. 8. Dendrogram showing the species relationships as determined from the matrix of correlation coefficients in Table 11. Species abbreviations are listed in the legend to Figure 1

in the construction of the dendrogram. *D. immigrans* joined the *D. melanogaster-D. simulans* cluster and, in addition, *D. emarginata* and *D. nebulosa* as well as *D. mulleri* and *D. mercatorum* were grouped when the r interval of +0.81 to +0.90 was considered. Subsequent clustering occurred in the +0.71 to +0.80, +0.51 to +0.60, and +0.31 to +0.40 intervals. The mean of all the r values involving *D. virilis, D. mulleri* and *D. mercatorum* with the remaining seven species, was -0.19, as indicated in Figure 8.

Table 6. Matrix of correlation coefficients for each species pair. See text for the method of computation. Species abbreviations are listed in the legend to Figure 1

	MEL	SIM	PAUL	WILL	NEB	EMAR	IMM	MULL	MERC	VIR
MEL	—									
SIM	> +0.99	—								
PAUL	+0.24	+0.21	—							
WILL	+0.21	+0.19	> +0.99	—						
NEB	+0.29	+0.29	+0.70	+0.69	—					
EMAR	+0.60	+0.59	+0.74	+0.76	+0.86	—				
IMM	+0.86	+0.85	+0.30	+0.28	+0.34	+0.75	—			
MULL	+0.47	+0.35	-0.24	-0.25	-0.08	+0.32	+0.77	—		
MERC	+0.08	+0.06	-0.54	-0.55	-0.42	-0.10	+0.41	+0.89	—	
VIR	-0.46	-0.45	-0.83	-0.84	-0.77	-0.68	-0.26	+0.40	+0.77	—

Discussion

Several observations need to be made concerning the immunological method used in this study before comparing the species relationships established from this examination of acid phosphatase-1, on the one hand, and from more traditional investigations on the other. First, it is preferable in immunological studies of protein variation to use antisera pooled from several rabbits in order to minimize the effects of inter-rabbit variation on antiserum specificity (see Champion, 1971a; Prager and Wilson, 1971). Indeed, when considering the results discussed above, one should take into account the possibility that the single antisera against the enzymes from *D. nebulosa, D. paulistorum* and *D. virilis* may not recognize all the determinants on the surfaces of the homologous acid phosphatases. Nevertheless, the two antisera against the enzyme from *D. melanogaster* produced almost identical results in the interspecific tests, both in terms of the rank order of species on the basis of immunological distance and the absolute values of those distances. Only the absolute immunological distances from *D. melanogaster* to *D. immigrans* and to *D. virilis* are significantly different in the two series of tests. Clearly, pooling the two antisera before performing the inhibition tests would not have altered the interspecific test results in any important way.

A second point about the method used in this study concerns the variation in the slopes of the regression lines of percent inhibition versus the log of the antiserum concentration. These slopes, together with their standard errors, are given in Tables 1-5. In microcomplement fixation studies, accurate measurement of immunological relatedness requires that the slopes of the regression lines of the percent complement fixed at the peak versus the log of the antiserum concentration be identical. Chi square tests for homogeneity of the slopes reported within each of Tables 1-5 indicate there are several significant differences. These are almost exclusively due to the steeper slopes of the regression lines falling within the interval between undiluted and a five-fold dilution of the antiserum. By choosing the antiserum concentration producing 50% enzyme inhibition as the basic datum for all our comparisons, however, the effect of these differences in slope on the results should be minimal.

A third desirable aspect in immunological studies on protein variation is "good" reciprocity. That is, the immunological distance from species X to species Y (i.e., when antisera against the protein from species X is cross-reacted with the enzyme from species Y) should equal the immunological distance from species Y to species X (when antiserum against the protein from species Y is cross-reacted against the enzyme from species X). Perfect reciprocity is almost never achieved (see Nei, 1977 and Champion et al., 1975, for a discussion). The "goodness of fit" of a set of results to perfect reciprocity can be estimated as a percent standard deviation (σ) according to Maxson and Wilson (1975) as:

$$\sigma = 100 \sqrt{\frac{1}{n} \sum_{i=1}^{n} \frac{(a_i - b_i)^2}{(a_i + b_i)}}$$

Where n = the number of rests and $a_i + b_i$ are the immunological distances of reciprocal tests for the i'th test. Table 7 presents the $a_i + b_i$ values for the reciprocal tests in our study. σ calculated for the seven reciprocal tests excluding those of *D. virilis* with *D. melanogaster*, is 21%. If the reciprocal tests between *D. virilis* and *D. melanogaster*,

Table 7. Immunological distances in the reciprocal tests in this study

Enzyme \ Antiserum	Anti-Neb	Anti-Paul	Anti-Mel #1	Anti-Mel #2	Anti-Vir
Neb		102	133	156	201
Paul	48		128	151	221
Mel	224	204			—*
Vir	172	209	284	—*	

*Cannot be estimated

for which immunological distances cannot be measured empirically, are simply given values of 300 (a presumed upper limit for immunological distances), σ, recalculated on the basis of nine tests, drops to 18%. These values of σ are not appreciably larger than the percent standard deviations reported in other studies (see Champion et al., 1975), and is less than that determined by Prager et al. (1976).

Finally, with regard to the statistical significance of the immunological distance differences, it should be noted that we have adopted a very conservative position, i.e., we require that there be no overlap between the maximum and minimum immunological distances of two species from the species providing the homologous value before that distance difference is considered significant. Nei (1977), in a recent paper, has also pointed out with data from microcomplement fixation tests that the empirical variance of a set of immunological distances is at least two times larger than the mean. It is interesting that the maximum and minimum limits surrounding our mean estimates of immunological distance, which directly reflect the experimental error in our method, generally span intervals which could have been generated by variances as large as those estimated by Nei. This, however, may be purely coincidental.

The phylogeny of the genus Drosophila is particular well understood, due in large part to the efforts of Sturtevant (1942), Patterson and Stone (1952) and Throckmorton (1962; 1974). Since there is a virtual absence of Drosophilid fossil data, the information has come almost exclusively from morphological and cytogenetical investigations. As was indicated in the Introduction, there are only a few studies on the evolution of specific Drosophila proteins. The information presented above on the immunologically detected differences in the structure of acid phosphatase indicates more studies of proteins can be useful in systematic and phylogenetic investigations of Drosophila. Thus, there is very close correspondence between the dendrogram generated from the acid phosphatase cross-reaction data (Fig. 8) and the dendrogram depicting the classical phylogenetic relationships between the ten species used in this study (Fig. 9). There are two exceptions, however which merit some discussion. First, the results of the immunological study join *D. emarginata* and *D. nebulosa*, whereas *D. nebulosa* is actually in the *willistoni* species group (see Fig. 9). Examination of Figures 4 and 5 clearly show that with the antiserum against the acid phosphatase from either *D. nebulosa* or *D. paulistorum*, *D. nebulosa* is "closer" to the other *willistoni* group species than it

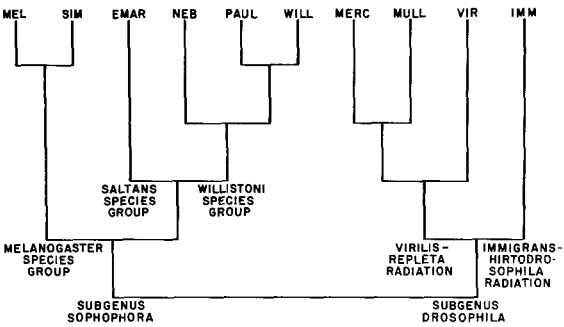

Fig. 9. Phylogenetic relationships of the species used in this study according to Throckmorton (1974). See Figure 1 legend for an explanation of the species abbreviations. No time scale is implied on the vertical axis

is to *D. emarginata.* The clustering of *D. nebulosa* with *D. emarginata* is probably due to the fact that in two of the five antiserum tests, the enzymes from *D. nebulosa* and *D. paulistorum* were the antigens. Thus, in the tests with the anti-*D. nebulosa* serum, the antiserum concentration necessary to inhibit 50% of *D. nebulosa*'s acid phosphatase (the homologous value) was rather low and, of course, much lower than that concentration inhibiting 50% of *D. paulistorum*'s enzyme. The opposite was true in the tests with the anti-*D. paulistorum* serum. In each series of tests, the heterologous value for *D. emarginata* was less than the homologous value, as expected. The reversal of the high and low relative values in the comparison of *D. paulistorum* and *D. nebulosa,* however, will tend to lower their correlation coefficient. This effect will be more profound than the effect of the two low heterologous values involving *D. emarginata* on the correlation coefficients between *D. emarginata* and *D. nebulosa* or *D. paulistorum.* In other words, the joining of *D. emarginata* to *D. nebulosa* in the dendrogram in Figure 8 is a result of a bias in the data, a bias which results from the use of antisera against the enzymes from two closely related species. The overall usefulness of the correlation coefficients in the construction of the dendrogram does not appear to be seriously jeopardized by this bias, however. In fact, *D. emarginata* is closely related to the *willistoni* group species (see Throckmorton, 1975) and Figure 8 shows this.

The second difference between the dendrograms in Figures 8 and 9 is more puzzling. *D. immigrans* is not even in the subgenus Sophophora, yet the results from the immunological tests with *D. immigrans* are highly correlated with those from tests with *D. melanogaster* and *D. simulans* (r = +0.86 and +0.85). In fact, were it not for these high r values, *D. immigrans* would have been "correctly" clustered with the species from the *repleta* radiation. Part of this correlation is due to the fact that all three species, *i.e., D. immigrans, D. melanogaster,* and *D. simulans* are immunologically quite distant from *D. paulistorum, D. nebulosa* and *D. virilis* (see Figs. 4, 5 and 6). Figure 7 clearly shows, however, that there is a surprisingly strong cross-reaction between the acid phosphatase from *D. immigrans* and both of the anti-*D. melanogaster* sera. This implies there are some biologically meaningful similarities between the acid phosphatases from the two species. The electrophoretic mobility of the enzyme from *D. immigrans* is different from those acid phosphatases from the other species of the subgenus Drosophila (see Fig. 1). It is, in fact, more electronegative like the enzymes from the Sophophoran species used in this study. It is worth pointing out that the surface amino acid residues contributing to net charge are likely to be part of one or more antigenic determinants. It may be, therefore, that the apparent

convergence in the immunological properties of the enzymes from *D. immigrans* and the *melanogaster* group species is due to their similarities in net charge. In this regard, results from tests with antisera against the enzyme from *D. immigrans* would be extremely interesting.

It is clear from the results presented above that acid phosphatase is not evolving at a constant rate in all the lineages of the genus Drosophila. If acid phosphatase had evolved at a constant rate in all the lineages, species from one subgenus or radiation should be immunologically equidistant from a species in another subgenus or radiation in tests with an antiserum against the enzyme from that latter species. Thus, the immunological distances of *D. mulleri* and *D. mercatorum* from *D. virilis* are not the same (see Fig. 6), even though *D. mulleri* and *D. mercatorum* diverged from a common ancestor *after* the line leading to *D. virilis* diverged from the *mulleri-mercatorum* lineage. Also, the acid phosphatase from *D. virilis* appears to have evolved more rapidly than the other *repleta* species' enzymes when one considers the results from the tests with the two anti-*D. melanogaster* sera (Fig. 7). The most striking example of an unequal rate of change of acid phosphatase can be seen in the *melanogaster* group species. Both the anti-*D. nebulosa* (Fig. 4) and the anti-*D. virilis* sera (Fig 6) barely cross-react with either the *D. melanogaster* or the *D. simulans* enzyme. Thus, according to the former test the enzymes from species of the subgenus Drosophila are immunologically closer to the *D. nebulosa* enzyme than are the acid phosphatases from *D. melanogaster* and *D. simulans*. And, in the latter test, i.e., with the anti-*D. virilis* serum, if the enzyme were evolving at a constant rate in the lineages of the subgenus Sophophora, the immunological distances of the *melanogaster* and *willistoni* group species from *D. virilis* should have been the same. This is certainly not the case.

In addition to having unequal evolutionary rates in different lineages in the genus Drosophila, acid phosphatase-1 has apparently evolved quite rapidly in the genus. A very rough estimate of an average rate can be obtained, even though the immunological distances determined above cannot be calibrated in terms of probable numbers of amino acid substitutions. Other studies involving the immunological technique of microcomplement fixation discussed above indicate there will be no cross-reaction between an antiserum and a heterologous antigen when there is approximately a 30-40% amino acid sequence difference between the protein used to elicit the antibodies and the heterologous protein (Prager and Wilson, 1971b; Champion et al., 1974). In the reciprocal tests involving anti-*D. virilis* and anti-*D. melanogaster* sera, very little or no cross-reaction was detected. The acid phosphatases from *D. virilis* and *D. melanogaster*, then, may have diverged to the extent that about 30% of their amino acid residues are different. The subunit molecular weight of this homodimeric enzyme is 55,000 daltons (MacIntyre, 1971). Since the average molecular weight of an amino acid residue is 110 daltons, the acid phosphatase gene produce may have approximately 500 residues. If 30% of these are different, then in each lineage, *i.e.*, in the one leading to *D. melanogaster* and in the one leading to *D. virilis*, some 75 amino acids may have been substituted, or approximately 16 substitutions per 100 residues (the latter number is 15 corrected for multiple changes; see Dickerson, 1971). Collier and MacIntyre (1977) have estimated, on the basis of the evolutionary rate of Drosophila α-glycerophosphate dehydrogenase, that the divergence leading to the present subgenera Sophophora and Drosophila occurred approximately 52 million years ago. If this estimate

is correct, acid phosphatase may be evolving at a rate of one amino acid substitution (per 100 residues) every 3.25 million years (52M.Y./16). The rate may, in fact, have been more rapid in the *D. melanogaster* lineage in light of the discussion above on unequal rates of acid phosphatase evolution. This estimate of a unit evolutionary period (UEP) of 3.25 M.Y. stands in marked contrast to α-glycerophosphate dehydrogenase which, within the genus Drosophila, undergoes a 1% sequence divergence every 29.4 million years.

The estimate of the acid phosphatase UEP depends upon an assumption that has not yet been verified. The assumption is that the virtual lack of cross-reactivity between *D. melanogaster* and *D. virilis* acid phosphatases would also be found if we had used the technique of microcomplement fixation. Clearly microcomplement fixation is a very sensitive immunological test, but it should be noted that our span of reliably measured immunological distances is equivalent to those obtained in microcomplement fixation studies (see Champion, et al., 1974). The technique used in this paper is also useful in that antisera against a pure protein are not required. The relationship between the two techniques with regard to accuracy and sensitivity, however, needs to be empirically examined on the same set of antigens.

A final warning about these results should be made. We have assumed that the differences in cross-reactivity of the acid phosphatases result from amino acid sequence differences. We have not, however, determined if and how much carbohydrate is present on acid phosphatase. Carbohydrate moieties can contribute to the antigenicity of glycoproteins. If sugars are part of the antigenic determinants of acid phosphatase, however, their variation has not affected the basic agreement between the dendrograms in Figures 8 and 9. Their presence in antigenic determinants would, however, result in an overestimate of the UEP.

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