EVOLUTION OF XANTHINE DEHYDROGENASE IN DROSOPHILA¹

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THE use of biochemical properties for classifying a given species and for determining its evolutionary relationships has developed very rapidly over the last decade, owing mainly to the ready availability of increasingly sophisticated biochemical techniques (see ALSTON and TURNER (1963) and BRYSON and VOGEL (1965) for the current biochemical and genetic achievements and views in the field of molecular evolution). Despite the wide use of *Drosophila* in genetic investigations, few applications have been made of biochemical characteristics in the study of the systematics or evolution of this organism. Studies involving pteridines have been reported (HUBBY and THROCKMORTON 1960; THROCKMORTON 1962a; THROCKMORTON and MAGALHAES 1962; HOENIGSBERG *et al.*, 1965); the evolutionary relationships between adult soluble proteins within the virilis group of *Drosophila* have been studied by vertical polyacrylamide gel electrophoresis (HUBBY and THROCKMORTON 1965); an inter-species survey has been described of late third-instar larval lymph protein content, as shown by starch gel electrophoresis, of seven species of *Drosophila* (DUKE 1966); an investigation based on polyacrylamide electrophoretic patterns of 10 enzymes and 11 proteins in *D. pseudoobscura* has evaluated the genetic variation which occurs within and between populations (HUBBY and LEWONTIN 1966; LEWONTIN and HUBBY 1966); polyacrylamide zymograms of amylase isozymes have been compared between different species of *Drosophila* (DOANE, personal communication). The sole use, however, of electrophoretic patterns might be misleading in providing definitive information on problems of evolution and taxonomy, because once the mobility of a protein shifts during evolution it is difficult to re-establish identity. It was decided, therefore, to study the evolution of a single enzyme type for which sensitive specific assays are readily available. The present paper describes an attempt to study the evolution of xanthine dehydrogenase (XDH) in *Drosophila* based on polyacrylamide gel patterns, serological relationships, and molecular weight comparisons (see GLASSMAN (1965) for a review of the genetic control of this enzyme).

MATERIALS AND METHODS

Drosophila stocks: The stocks used in this investigation were from the following sources.

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Dr. H. Carson, Washington University, St. Louis:

D. americana texana (A-12, Astor, Florida)—strain 2, *D. buzzatii* (B1-7, Israel), *D. immigrans* (119.5, Mt. Tantalus, Oahu, Hawaii), *D. mercatorum* (RI, Rochester, N.Y.), *D. paranaensis* (EL-3, El Real, Panama), *D. robusta* (A-7 = Astor, Florida).

Hastings, Michigan:

D. americana americana—strain 1.

Dr. W. Heed, University of Arizona:

D. hamatofila (A23.2)—strain 1, *D. hamatofila* (A105.1)—strain 2, *D. longicornis* (A22.2)—strain 1, *D. nigrohydei* (A39.9), *D. sucinea* (A105.2).

Institute of Animal Genetics, Edinburgh:

D. melanogaster (*Pacific*) (Pac).

Marriton, Arkansas:

D. americana texana—strain 1.

Dr. L. Mettler, North Carolina State University:

D. arizonensis, *D. funebris*, *D. hydei*, *D. immigrans*, *D. mulleri*, *D. pseudoobscura*, *D. ritae*, *D. robusta*.

Oak Ridge National Laboratory:

D. melanogaster (Oregon-R).

Pennsylvania State University:

D. melanogaster (*Pm;Sb/Xa*).

Dr. H. Stalker, Washington University, St. Louis:

D. euronotus (S-45b)—strain 1, *D. euronotus* (S-Eu-Pal 21)—strain 2, *D. melanica* (S-30 dhl), *D. melanura* (S-PR2464), *D. micromelanica* (S-SM-1), *D. nigromelanica* (S-TH 703F), *D. paramelanica* (S-28-12).

Yale University: *D. littoralis*, *D. simulans*, *D. virilis*.

Electrophoresis: Polyacrylamide gel electrophoresis at pH 9 and detection of XDH on the gel was carried out by the method of YEN and GLASSMAN (1965). Depending on the species, two to six freshly emerged flies were used in each electrophoretic sample. A mixed sample of F (fast) and S (slow) *D. melanogaster* XDH types was included on all gels as a reference. The F band was given a mobility value of 100 and the mobilities of all other bands were related to this value. The relative mobility of the I (intermediate) band is 98 and that of the S band 96. The distance of migration was measured from the origin to the center of the enzyme band as many bands were of variable width. The XDH bands in each species were identified by incubation of similar gels with and without hypoxanthine.

Serological Analysis: Antiserum to partially purified XDH from *D. melanogaster* (Pac) was prepared and titred by the method described by KARAM (1965). XDH from Pac flies is the I type which is the most prevalent variant found in *D. melanogaster* (YEN and GLASSMAN 1965). The antiserum was then used in two ways to study serological relationships.

a) *The percentage inhibition of XDH activity* by equivalent amounts of antiserum made with XDH from *D. melanogaster* was measured in the following fashion. Crude fly extracts were prepared by homogenization of adult flies in one volume (w/v) of 0.05 M Tris-(hydroxymethyl)-aminomethane (Tris, Sigma) containing 0.001 M disodium ethylenediamine tetra-acetate (EDTA, Fisher), pH. 8.0. The homogenate was centrifuged at $30,000 \times g$ for 30 minutes, the supernatant filtered through glass-wool, and the filtrate then passed through a (1.1 $\times$ 25 cm) G-25 Sephadex column. The elute fractions containing XDH were adjusted to 6 mg protein per ml with the Tris-EDTA buffer. Protein was measured by a modification of the method of LOWRY *et al.* (1951). Four tenths ml of this crude extract was incubated for 10 hours in the cold with 0.2 ml antiserum. The antigen-antibody precipitate was removed by centrifugation at $21,700 \times g$ in the Misco Centrifuge for three minutes. The mixtures were assayed for XDH before and after incubation by the fluorometric method of GLASSMAN (1962). In all experiments a control of non-immune serum was substituted for the antiserum to measure the stability of the enzyme during the incubation period. The level of inhibition for *D. melanogaster* was fixed at 100% and all other values related to it.

b) *The amount of cross-reacting material* (CRM) to Pac XDH from *D. melanogaster* was

estimated in the Pac stock and in six other species of *Drosophila* by the method of GLASSMAN and MITCHELL (1959) as modified by KARAM (1965). Crude G-25 Sephadex fly extracts were adjusted to 3 mg. protein per ml. XDH reference enzyme for use in the CRM test was prepared by purification method B of KARAM (1965). The CRM value for *D. melanogaster* was adjusted to 100% and all other values related to this level.

Sucrose Gradient Centrifugation: The optical density at 280 m μ of crude G-25 Sephadex extracts was adjusted to 40 to 50 units per ml. One ml of extract was layered on 46 ml of a linear (5-20%) sucrose gradient and centrifuged in the SW-25.2 rotor of the Spinco Model L-2 ultracentrifuge for 20 hours at 100,000 g. Fractions of 1.4 mls were collected and assayed for XDH.

RESULTS

Figure 1 shows the electrophoretic patterns of xanthine dehydrogenase in 29 species of *Drosophila* from nine different groups. The patterns of the species tend to fall loosely into those groups recognized by taxonomists on the basis of chromosome homologies and various other criteria (PATTERSON and STONE 1952; THROCKMORTON 1962b). The mobilities of the XDH from the different species, particularly those of the melanogaster, the virilis and the repleta groups, differ quite considerably from one another.

The kind of XDH band obtained under a given set of conditions may, possibly, be another expression of evolutionary divergence between the species. Thus, the XDH patterns of the repleta group were either not detectable under the electrophoretic conditions, which were originally worked out for the *D. melanogaster* XDH, or the bands were diffuse and streaked indicating that the electrophoretic conditions suitable for the *D. melanogaster* XDH are not suitable in this case. The same was true for XDH from *D. polychaeta* of the polychaeta group, which gave a pattern that was completely streaked from the origin to the buffer front. Some other species also exhibited XDH bands that migrated more diffusely than those of the melanogaster group.

In a few individuals of certain species a minor XDH band of low staining intensity appeared in addition to the major band (see Figure 1). In the case of *D. robusta*, however, the majority of individuals tested had this additional band. The significance of these minor bands is not yet clear.

Figure 2 shows a scheme of evolution of species within the melanica group based on salivary gland studies (STALKER 1966). It is of interest to note that the electrophoretic mobilities get progressively slower in those species of later evolutionary lineage. STALKER (1966) maintains that *D. robusta* is closely related to *D. nigromelanica* and *D. micromelanica*. It may be significant that the electrophoretic mobilities of their XDH molecules are also very similar. It should be pointed out, however, that the *D. sucinea* and *D. immigrans* XDH types are also closely related in mobility to that of *D. nigromelanica*. (See Figure 1). Additional information to electrophoresis is, therefore, required.

For this reason, certain serological relationships were tested. In Figure 3a the percent inhibition of XDH from various species by antiserum to *D. melanogaster* XDH is compared to the amount of crossreacting material (CRM) to the same *D. melanogaster* antiserum. In both tests the value for *D. melanogaster* was fixed at 100 and all other values were related to this level. It can be seen that there is

FIGURE 1.—Diagram of the relative electrophoretic mobilities of xanthine dehydrogenase in polyacrylamide gel from 27 species, comprising nine species groups of the genus *Drosophila*. The fast (F) *melanogaster* band was given a mobility value of 100% and all other mobilities were related to it. The group identities are given at the top of the diagram, and listed underneath are the members of each group in order of electrophoretic mobility. Where more than one strain of a species was studied, the species name is numbered.

FIGURE 2.—Evolutionary relationships within the *melanica* group based on salivary gland studies (after STALKER 1966). The relative electrophoretic value for each species is shown in brackets. Arrows indicate the direction of evolution.

a direct relationship between the two tests. As expected, *D. melanogaster* XDH was inhibited most, and cross-reacted to the greatest extent with *melanogaster* antiserum. The antigenic relatedness between XDH of the species shown in Figure 3a corresponds with the scheme of evolutionary relationships in the genus *Drosophila* described by THROCKMORTON (1962b), although according to PATTERSON and STONE (1952), *D. virilis* is more closely related to *D. melanogaster* than is *D. funebris*.

A comparison of the percent inhibition by antiserum with the relative electrophoretic mobilities (Figure 3b) indicates some relationship between these parameters. *D. simulans* XDH is similar in electrophoretic mobility to that of *D. melanogaster* and is greatly inhibited by the *melanogaster* antiserum. The XDH from *D. funebris*, *D. virilis*, and *D. hydei* was inhibited in the same order as the relative rates of electrophoretic mobilities. The only species not to fall on the curve was *D. pseudoobscura*. This occurred because the species had an electrophoretic band faster than *D. melanogaster*. If we had prepared antiserum to XDH from *D. pseudoobscura*, rather than to *D. melanogaster*, it is most probable that a single curve would have been obtained in Figures 3b and 3c. These data suggest that there is a relationship between the electrophoretic migration of XDH and the serological properties of the molecule.

It would be expected that, since CRM and the percent inhibition by antiserum are related properties of XDH (Figure 3a), similar results for both tests should be obtained. In Figure 3c it can be seen that the CRM measurements closely approximate those obtained in the percent inhibition experiment.

Large electrophoretic differences in polyacrylamide gels such as those observed for XDH from *D. melanogaster* (Pac), *D. funebris*, and *D. hydei* can be due to large changes in molecular size. It can be seen, however, in Figure 4 that the molecular weights as indicated by sucrose density gradient centrifugation are very similar in all three species.

DISCUSSION

Xanthine dehydrogenase has many advantages for the study of molecular evolution in *Drosophila*. A sensitive fluorometric assay is available (GLASSMAN 1962) and methods of acrylamide gel electrophoresis (YEN and GLASSMAN 1965) and assay for crossreacting material (GLASSMAN and MITCHELL 1959) have been described. In addition, XDH from *D. melanogaster* has been shown to vary little with respect to electrophoretic mobility and kinetic properties as a result of mutation at the *rosy* locus, the structural gene for XDH (YEN and GLASSMAN 1965, 1967). These studies revealed the closeness of the electrophoretic mobility of XDH in 56 strains of *D. melanogaster* and also demonstrated the lack of isozymes under the conditions used. XDH in this respect is in direct contrast, for example, to malate dehydrogenase which exhibits a large amount of both inter- and intra-species variation (HUBBY, personal communication) and to alcohol dehydrogenase where three widely separated isozyme systems have been described (GRELL *et al.* 1965).

The goal of the present investigation is concerned with the evolutionary

FIGURE 3.—Correlation of the serological relationships between certain species of *Drosophila* and their relative electrophoretic mobilities. (a) The relationship of the percent inhibition of xanthine dehydrogenase by XDH antiserum to the amount of crossreacting material in six species of *Drosophila*. The *D. melanogaster* value in each test was adjusted to 100% and all other values related to this level. (b) The relationship between the relative electrophoretic mobilities in polyacrylamide gel and the level of inhibition of XDH by *D. melanogaster* XDH antiserum in six different species. (c) Relationship between the relative electrophoretic mobilities and the level of CRM in seven different species.

changes of a single enzyme. Figures 5a and 5b show how these changes compare with two models of evolutionary relationships between species groups in the genus *Drosophila* that are based on morphological and cytological studies (see Figure 34 in PATTERSON and STONE 1952, and Figure 53 in THROCKMORTON 1962b). Because of the possible variation in the absolute time of the separation of one species from another, it is difficult to draw comparisons between these models and the evolutionary changes of XDH. For example, the two large subgenera, *Sophophora* and *Drosophila*, diverged at a relatively early stage from one another and it is possible that certain species within one group are equally separated in time from any species in the other group (THROCKMORTON, personal communication). Certain trends, however, do emerge. There is, with certain exceptions, a tendency for the XDH molecule to become electrophoretically slower in those species of later evolutionary lineage. This can be more easily observed in the recently formulated model of THROCKMORTON (Figure 5a). The model places *repleta* as a terminal group rather than giving rise to other groups as in the model of PATTERSON and STONE (Figure 5b). The XDH of the *repleta* group would

FIGURE 4.—Molecular weight comparison by sucrose density gradient centrifugation of xanthine dehydrogenase in *D. melanogaster* (Pac), *D. funebris* and *D. virilis*.

also appear to have terminal electrophoretic and serological values (see Figure 3). This model, in addition, places the derivation of the immigrans group nearer to the stem population of the sub-genus *Drosophila* than does the model of PATTERSON and STONE. The high electrophoretic and CRM values of the immigrans XDH are similarly close to those values so far obtained from members of the sub-genus *Drosophila*. Both THROCKMORTON's model and STALKER (1966) indicate the closeness of the robusta to the melanica group and this is reflected in their electrophoretic values for XDH (Figure 5a). The two groups, however, have higher electrophoretic values than would be expected from either model, assuming that the XDH bands from species of later evolutionary lineage do, in fact, get electrophoretically slower in our system. We have not yet been able to test either of these groups for CRM. The evolutionary tendencies of XDH, as indicated by the present investigation appear to correlate more closely, therefore, with THROCKMORTON's model of phylogenetic relationships although it is unclear why the enzyme should tend to get electrophoretically slower in those species of later evolutionary lineage.

FIGURE 5.—Taxonomic relationships in *Drosophila*. (a) Modified scheme of THROCKMORTON (1962b). (b) Modified scheme of PATTERSON and STONE (1952). In both cases arrows indicate the direction of evolution from the primitive ancestor. The relative mobilities and CRM values, where studied, are shown in brackets.

Pyridoxal oxidase and aldehyde dehydrogenase in *D. melanogaster* are enzymes affected by some of the genes that also affect XDH (see GLASSMAN 1965, for a review of this problem). Preliminary experiments with aldehyde dehydrogenase using techniques similar to those of J. B. COURTRIGHT (personal communication), indicate that interspecies relationships of the electrophoretic patterns are not as clear as for XDH. This may mean that the correlation between electrophoretic mobility and serological values for XDH may not hold true for certain other enzymes. We shall have to withhold judgment, however, until serological analysis is carried out. Thus, the importance of using several criteria of molec-

ular relatedness (i.e., electrophoretic mobility, molecular weight and serological analysis) is again emphasized for these types of studies.

It is still unclear what selective forces are involved in the evolution of these different XDH phenotypes. The development of a minor enzyme band, of the type observed in *D. pseudoobscura*, *D. simulans*, etc., (see Figure 1) may constitute at least one of the mechanisms of evolutionary change, the band being subsequently selected for so that ultimately it becomes the only one present. This might be the case for the minor XDH band in *D. robusta*. STALKER (1966) has placed *D. robusta* close to those species of the melanica group of early evolutionary lineage, and in Figure 1 it can be observed that the respective electrophoretic mobilities are also very similar. Furthermore, the minor band in *D. robusta* is close in mobility to those melanica species of later evolutionary lineage. It can be speculated, therefore, that the XDH of those melanica species of later evolutionary lineage may have been derived from the early type through a similar mechanism. It is of interest that aldehyde dehydrogenase in *D. melanogaster* has a similar electrophoretic mobility to a minor XDH band which appears on our gels under certain conditions and might represent another instance of this phenomenon.

The molecular weight comparisons of XDH types with widely divergent electrophoretic mobilities show little, if any, difference (see Figure 4). Presumably, therefore, the differences described in this paper are not due to changes in molecular size, but rather are due to amino acid substitutions, alterations in the co-factors within the XDH molecule, or conformational changes. The kinetics of the electrophoretic variants of XDH of *D. melanogaster* have been studied by YEN and GLASSMAN (1967). They concluded that no significant differences existed in the catalytic functions of the variants. This rules out variation of these catalytic functions being a selective force here, although untested factors might be operating.

SHAW (1965) has proposed the term "isokinetic" to denote variant enzyme forms which exhibit no detectable differences in catalytic activity. He points out that alterations in the molecule may affect other properties than catalysis. In addition, SHAW proposes that mechanisms other than natural selection could underlie those molecular alterations. In the case of XDH we have an enzyme which appears to be electrophoretically conservative on the intraspecies level, at least within *D. melanogaster*, and highly divergent on the interspecies level. This could mean that natural selective forces of some sort are in operation. Clarification of these problems will require an increasingly refined biochemical approach using many more species, with the determination of amino acid and nucleotide sequences being among the ultimate goals.

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SUMMARY

Significant variations in polyacrylamide gel electrophoretic patterns and sero-

logical relationships of xanthine dehydrogenase (XDH) have been observed between 29 species from nine different species groups of the genus *Drosophila*. These differences are not due to large changes in molecular size.—There is a tendency for the XDH molecule from species of early evolutionary lineage to migrate more rapidly during electrophoresis than there is from those of later lineage. In addition, a direct relationship has been established between the degree of electrophoretic mobility and the antigenic properties of the enzyme from seven different species. The significance of these observations is unclear. The data correspond more closely with THROCKMORTON's scheme of phylogenetic relationships based on morphological and cytological studies within the genus *Drosophila* than with the scheme outlined by PATTERSON and STONE.

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