Biochemical and Immunological Studies on an α-Glycerophosphate Dehydrogenase From the Tephritid Fly, *Anastrepha suspensa*

Ranjit Sarma, G. Barrie Kitto, Stewart Berlocher, and Guy L. Bush

Clayton Foundation Biochemical Institute, Department of Chemistry, The University of Texas, Austin (R.S. Deceased, G.B.K.); Department of Entomology, University of Illinois, Urbana (S.B.); Department of Zoology, Michigan State University, East Lansing (G.L.B.)

A rapid and efficient procedure has been developed for the purification of α-glycerophosphate dehydrogenase from the tephritid fly *Anastrepha suspensa*. This procedure is applicable to the isolation of the enzyme from other tephritids. The *A. suspensa* α-glycerophosphate dehydrogenase is dimeric with a molecular weight of 70,000 and a subunit molecular weight of 35,000. The pH optimum of the enzyme is 7.0. The amino acid composition is compared with that of other α-glycerophosphate dehydrogenases. By means of the quantitative microcomplementation fixation procedure the *A. suspensa* α-glycerophosphate dehydrogenase is compared immunologically to a variety of other tephritid and dipteran α-glycerophosphate dehydrogenases.

Key words: tephritid, immunological studies, biochemical studies

INTRODUCTION

The family of true fruit flies, the Tephritidae, are of major economic importance worldwide. The family includes, for instance, *Rhagoletis pomonella*, the apple maggot; *Rhagoletis cingulata*, a major cherry pest; *Dacus* species, which cause extensive damage to citrus and olive crops; and the polyphagous *Ceratitis capitata*, the Mediterranean fruit fly, which, in addition to attacking citrus, infests coffee, peach, and guava. At least 15 laboratories

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Address reprint requests to G. Barrie Kitto, Clayton Foundation Biochemical Institute, Department of Chemistry, The University of Texas, Austin, TX 78712.

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around the world are now using or developing biological control programs, such as sterile insect release, for controlling tephritid flies.

Little is known about the phylogenetic relationships of the Tephritidae, so it has been difficult to assess the likelihood that a trait useful for pest management in one group will be found in another. Also, we know very little about the evolution of their host plant utilization. For instance, some genera now attack similar hosts, yet we are not sure that this similarity in behavior and ecology is the result of convergence or that the genera, some of which are now placed in separate tribes or subfamilies, actually share the trait through descent from a common ancestor. In order to establish a better understanding of phylogenetic relationships we have undertaken a broad study of protein evolution in the Tephritidae, using as a primary tool the immunological technique of microcomplement fixation [1,2].

The work described in this paper concerns Anastrepha suspensa, the Caribbean fruit fly, a near relative of another major pest, Anastrepha ludens, the Mexican fruit fly. Anastrepha suspensa is one of several fruit flies indigenous to the West Indies, the larvae of which infest a variety of tropical and subtropical fruits [3]. The preferred host plants of A. suspensa are common guava, surinam cherry, roseapple, peach, and tropical almond. A. suspensa has existed continuously in Florida since 1965, and it is viewed with some concern as a potential pest of commercial citrus.

In order to assess the genetic relationships of A. suspensa to other tephritid species we have chosen to isolate the extramitochondrial enzyme L-α-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase E.C. 1.1.1.8), to make rabbit antisera directed against this enzyme, and to use the microcomplement fixation procedure to compare the A. suspensa enzyme with homologous enzymes from other species.

α-Glycerophosphate dehydrogenase was chosen for this study for several reasons. First, it is present in high concentrations in the thoracic muscles of many Diptera [4] and thus offers the possibility of developing a rapid procedure for isolating the enzyme from the small samples of tephritid species available from field collections. Second, acting in concert with the flavin-linked mitochondrial L-glycerol-3-phosphate oxidase, this dehydrogenase plays a crucial role in the flight muscle metabolism of these insects [5-7]. It has been shown [8] that mutants of Drosophila melanogaster lacking cytoplasmic α-glycerophosphate dehydrogenase activity are unable to fly for extended periods. The third criterion was that we wanted to use an enzyme that was evolving relatively slowly so that we could utilize immunological testing across a broad range of species. By means of immunological procedures Brosemer et al. [9] and Fink et al. [10] found that α-glycerophosphate dehydrogenase structure was strongly conserved during the evolution of the Hymenoptera. Collier and MacIntyre [11] have shown that antisera against drosophilid α-glycerophosphate dehydrogenases will cross-react with this enzyme from a variety of dipteran families. These data suggested that α-glycerophosphate dehydrogenase should be well suited for examining the broader phylogenetic relationships within the Tephritidae and to explore the relationships of this family to other Diptera.

The microcomplement fixation procedure was chosen since extensive previous studies have shown that the immunological differences between ho-
mologous proteins as measured by this technique are strongly correlated with sequence differences between the proteins [12]. The studies by Collier and MacIntyre [11], Brosemer et al. [9], Fink et al. [10], and Beverly and Wilson [13] have clearly demonstrated the utility of the microcomplement fixation technique for insect systematics.

MATERIALS AND METHODS

The Caribbean fruit fly was mass-reared and supplied frozen by Dr. Milton Huettel of USDA-ARS, Insect Attractants, Behavior and Basic Biology Laboratory in Gainesville, FL. DHAP, GP, β-NAD, β-NADH, DTT, NBT, PMS, BSA, ammonium persulfate, glycine, TRIS, and rabbit muscle α-glycerophosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO. Affi-gel Blue, acrylamide, BIS, sucrose, and SDS were obtained from Bio-Rad Laboratories, Richmond, CA, and Sephacyrl S-200 superfine preswollen gel was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. For microcomplement fixation experiments, hemolysin was obtained from Baltimore Biological Laboratories, Baltimore, MD, complement serum (lyophilized) was obtained from Colorado Serum Co., Denver, and citrated sheep red blood cells were obtained locally. All reagents for buffers, potassium phosphate (mono- and dibasic), sodium chloride, sodium borate, boric acid, EDTA, sodium citrate, and sodium barbitol were purchased from Fisher Scientific Co., Houston, TX. Other chemicals were of reagent grade. For antiserum production New Zealand white rabbits were obtained locally.

Routine α-GPDH assays were done at room temperature (23°C) by measuring the oxidation of NADH at 340 nm spectrophotometrically. Standard assays were performed at 23°C in 0.9 ml of 0.1 M Tris-HCl buffer, pH 7.8, 0.1 mM DTT: 10 μl NADH (102 μM) and 100 μl DHAP (400 μM) and initiated with 10 μl of enzyme, appropriately diluted, to give a change in absorbance of 0.05–0.20 per min. One enzyme unit is defined as the amount of enzyme required to cause a rate of oxidation of 1.0 μmol of NADH per min. Protein concentrations were determined at 280 nm, as described by Layne [14]. The pH optimum for the enzyme was determined in buffers containing 0.02 M each of sodium citrate, sodium barbitol, monobasic potassium phosphate, and boric acid. The solution was adjusted to the desired pH levels with either 6 N HCl or 6 N NaOH.

Analytical polyacrylamide disc gel electrophoresis was carried out by the method of Ornstein [15] and David [16]. Starch gel electrophoresis was performed as described by Bush and Huettel [17] with a Tris-citrate buffer [18] at pH 8.6. Regions of enzymatic activity were located on the starch and

*Abbreviations: Affi-gel Blue = affinity chromatography gel; BIS = n, n'-methylene-bis-acrylamide; BSA = bovine serum albumin; DTT = DL-dithiothreitol; DHAP = dihydroxyacetone phosphate; EDTA = ethylenediamine tetraacetate; disodium salt; GP = DL-α-glycerophosphate; α-GPDH = α-glycerophosphate dehydrogenase; MC/F = Microcomplement fixation; β-NAD = β-nicotinamide adenine dinucleotide, reduced form; NBT = nitro blue tetrazolium, crystalline form; PMS = phenazine methosulfate; SDS = sodium dodecyl sulfate; TRIS = Tris (hydroxymethyl) aminomethane.
the polyacrylamide gels using a staining method specific for a α-GPDH [17,19]. The general protein stain was Coomassie brilliant blue (R-250).

The molecular weight of the enzyme was estimated by gel filtration using the procedure outlined by Murphy et al. [20]. The subunit molecular weight of the enzyme was determined by the method of Weber and Osborn [21] except that the protein samples were heated in boiling water for 2 min rather than incubated at 37°C for 2 h.

The amino acid composition of the protein was determined according to the procedure of Moore et al. [22]. Standard 24-h hydrolyses using 6 N HCl at 110°C were carried out on duplicate samples of the pure enzyme. Analyses were performed using a Beckman Model 121 automatic amino acid analyzer. Standards were prepared using a Beckman standard amino acid calibration mixture with an overall concentration for all standard amino acids of 0.1 μmol/ml.

Antisera to the purified α-GPDH were produced as follows. The initial injection contained approximately 100 μg of purified protein (=0.1 ml), emulsified with an equal volume of Freund's complete adjuvant and was administered in two areas, one-half subcutaneously and the other half intramuscularly in the thigh. All of the following injections (50 μg) were administered subcutaneously. Injections were given at 2-week intervals, and bleedings were taken at approximately one month intervals. At the end of 4 months the rabbits were given booster shots containing 500 μg pure protein, intravenously, in the ear. Four booster shots were given at weekly intervals, and a final bleeding was taken. The precipitin ring test as described by Brown [23] was used as qualitative method to observe production of antisera to purified α-GPDH during the immunization schedule. Immunodiffusion tests were done using the Ouchterlony double diffusion method [24].

Immunoinactivation studies were carried out by the procedure of Niesel et al. [25]. The inactivation of the purified α-GPDH was examined by incubating aliquots of enzyme with increasing antiserum concentrations in a final volume of 0.1 ml for 20 min at room temperature. The buffer used was 50 mM sodium phosphate, pH 7.8. Microcomplement fixation studies were performed as outlined by Wilson and coworkers [2,26], and the index of dissimilarity and the immunological distance were calculated by the method of Champion et al. [2].

Enzyme Purification

Several techniques were examined for developing an efficient and rapid purification procedure for tephritid α-glycerophosphate dehydrogenases. The following protocol has proven most effective for A. suspensa and other tephritid species. Approximately 50 g of frozen A. suspensa flies were homogenized for 5 min at top speed in a blender with 150 ml of ice-cold 0.025 M potassium phosphate buffer, pH 7.4, containing 0.5 mM EDTA, 1 mM DTT, and 0.1 mM KCN. The crude homogenate was then centrifuged at 20,000g for 15 min at 4°C. Powdered ammonium sulfate was added to the supernatant to give 40% saturation while maintaining the pH at 7.5 by addition of 10% ammonium hydroxide. After 1 h, the cloudy suspension was centrifuged at 20,000g for 15 min. The precipitate was discarded. The supernatant solu-

tion was brought to 0.8 M ammonium sulfate, and after standing overnight the precipitate was collected by centrifugation at 20,000g for 1 h. The precipitate was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM DTT, 1 mM EDTA, and 0.1 M ammonium sulfate, and dialyzed overnight against the same buffer. The dialyzed enzyme was then centrifuged at 20,000g for 1 h, and the supernatant was used for further purification.

The enzyme was further purified by chromatography on a column (100 mm x 2 cm) of DEAE-Sephadex A-50 equilibrated in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 0.1 M ammonium sulfate. The enzyme was eluted with a linear gradient of 0.1 M to 0.8 M ammonium sulfate in the same buffer. The eluate was monitored at 280 nm, and fractions containing enzyme activity were collected and concentrated by ammonium sulfate precipitation. The concentrated enzyme was further purified by gel filtration on a column (2.6 x 80 cm) of Sephadex G-100 equilibrated in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM DTT. The enzyme was eluted with the same buffer, and fractions containing enzyme activity were collected and concentrated by ammonium sulfate precipitation. The final preparation of the enzyme was homogeneous.

Immunology

Double diffusion and immunodiffusion tests for antibody production were performed. All sera were tested and sera titers were determined. Enzyme and bleeds were examined. The initial production had a titer of 5000, with rising to a titer of 100,000 from this final bleed. All sera were incubated with the enzyme used in the immunization experiments. This serum was used to prepare antisera to glycerophosphate dehydrogenase. A solution of the enzyme was prepared (100 μg/ml) in 0.1 M potassium phosphate buffer (pH 7.0), and 0.1 ml was added to 0.1 ml of serum, which was incubated for 1 h at 37°C. A precipitate was obtained, which was discarded. The supernatant was used for further purification.

RESULTS

In preliminary experiments, tephritid species of the genus A. were used as a source of the enzyme. The tephritid species contained the enzyme activity, and the
tion was brought to 70% saturation in ammonium sulfate in the same manner, and after 1 h it was centrifuged at 20,000g for 15 min.

The 40-70% precipitate was dissolved in a minimum volume of 0.025 M potassium phosphate buffer, pH 7.45, containing 1 mM DTT and 1 mM EDTA. The specific activity of the α-glycerophosphate dehydrogenase at this stage was three to four times that found in the crude extract. The sample was then dialyzed against four changes of the above buffer at 4°C over a period of 4 h. The dialyzed enzyme was then applied to an Affi-gel Blue affinity column (100 ml bed volume), which had been equilibrated with the dialysis buffer. The column was washed with this buffer until the absorbance at 280 nm dropped below 0.1. The α-glycerophosphate dehydrogenase was eluted with two bed volumes of the same buffer with 10 mM NAD added. Recovery of enzyme activity at this stage was approximately 60% of that found in the crude extract, and the specific activity was increased about 100-fold. Enzymatically active fractions from the Affi-gel Blue column were pooled and then concentrated to approximately 5 ml by ultrafiltration, using an Amicon Diaflo concentrator with an Amicon PM-10 membrane. The concentrated sample was then applied to a Sephacryl S-200 Superfine gel filtration column (2.6 × 80 cm) that was equilibrated and eluted with 0.05 M sodium borate buffer, pH 7.0, containing 0.1 M sodium chloride and 1 mM EDTA, using reverse flow at 4 ml/h at 4°C. The enzymatically active fractions were pooled and concentrated as previously described. At this point the α-glycerophosphate dehydrogenase was judged by electrophoretic techniques to be homogeneous.

Immunology

Double diffusion in agar was used as a qualitative means of monitoring antibody production during the extended antigen injection schedule. Antiserum titers were quantitated by the MC'F procedure, and the precautions of Wilson and coworkers were followed [2,27,28]. When three consecutive bleedings showed the same antibody titer, it was assumed that antisera production had reached a plateau, and a final bleeding was taken. Antisera from this final bleeding were used in all further studies. The primary antiserum used in our work (RS-GPD-12/S) had a titer of 4,019 for 50% complement fixed. This titer is comparable to the figures reported for other insect α-GPDH antisera [10,11]. For the immunoinactivation of purified A. suspensa α-glycerophosphate dehydrogenase by the rabbit antisera, equal aliquots of the enzyme were incubated with increasing concentrations of the antisera (0-10 μl), in a final volume of 0.1 ml, for 20 min at room temperature. The buffer was 50 mM sodium phosphate, pH 7.8.

RESULTS

In preliminary experiments, prior to enzyme purification, 40 individual specimens of A. suspensa were examined by starch gel electrophoresis. The gels were stained for protein and for α-glycerophosphate dehydrogenase activity, and the zymograms gave a single band of identical mobility for all
individuals examined, thus indicating the presence of only a single genotype for this enzyme in our mass-reared stocks.

**Enzyme Purification**

The procedure that we have developed for the purification of α-glycerophosphate dehydrogenase from *A. suspensa* is detailed in "Materials and Methods." Adoption of an affinity chromatography step, using Affigel-Blue and elution with NAD, provided a very effective separatory tool. A typical elution profile is shown in Figure 1. The final step in purification uses gel filtration to remove contaminants of both higher and lower molecular weights than the α-glycerophosphate dehydrogenase. An elution profile from this column is shown in Figure 1. A typical overall yield was 30-50%, with a final specific activity of 30 enzyme units/mg, representing an approximately 160-fold purification. The overall results of a typical purification run are shown in Table 1.

**Enzyme Characterization**

The purified *A. suspensa* α-glycerophosphate dehydrogenase was examined by analytical polyacrylamide disc gel electrophoresis at pH 9, using a range of protein concentrations. Duplicate gels were stained for protein and for α-glycerophosphate dehydrogenase activity, and single bands of identical mobility were obtained in each case, demonstrating the homogeneity of the samples. An approximate measure of the molecular weight of the enzyme was obtained by gel filtration on a Sephacryl S-200 column (0.9 × 50 cm) (Fig. 2). Samples of both rabbit muscle and *A. suspensa* α-glycerophosphate dehydrogenase.

![Fig. 1. Affinity chromatography of *A. suspensa* α-glycerophosphate dehydrogenase on AffiGel Blue (100–200 mesh). The enzyme solution, in 0.025 M potassium phosphate buffer, pH 7.45, containing 1 mM dithiothreitol and 1 mM EDTA, was applied to a column with a bed volume of 100 ml and eluted with the same buffer. At the point indicated, 10 mM NAD was added to the eluting buffer. The temperature was 4°C; ○○, enzyme activity; ●●, absorbance, 280 nm.](image)

**Table 1. Purification of *A. suspensa* α-glycerophosphate dehydrogenase**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>(NH₄)₂SO₄ 40% saturation</td>
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<tr>
<td>Affi-gel chromatography</td>
<td>Sephacyrl S-200 chromatography</td>
</tr>
</tbody>
</table>

![Fig. 2. Chromatography of *A. suspensa* α-glycerophosphate dehydrogenase on Sephacryl S-200 Superfine. The enzyme solution was in 0.1 M Tris-HCl buffer, pH 7.0, containing 1 mM dithiothreitol and 1 mM EDTA. The elution was at 4°C. ○○, enzyme activity; ●●, absorbance at 280 nm.](image)

**Immunology**

Initial experiments were carried out using different enzymatic activities in the preparation.
TABLE 1. Purification of *Anastrepha suspensa* α-Glycerophosphate Dehydrogenase

<table>
<thead>
<tr>
<th>Steps</th>
<th>Volume (ml)</th>
<th>Total enzyme units</th>
<th>Total protein</th>
<th>Specific activity (units/mg protein)</th>
<th>Percent yield</th>
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<td>(NH₄)₂SO₄, 40–70%</td>
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<td>Affi-gel chromatography</td>
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<tr>
<td>Sephacryl S-200 chromatography</td>
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<td>155</td>
<td>10.4</td>
<td>30.9</td>
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Fig. 2. Chromatography of *A. suspensa* α-glycerophosphate dehydrogenase on a Sephacryl S-200 Superfine column (2.6 x 80 cm). The enzyme was eluted with a 0.05 M sodium borate buffer, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA at a flow rate of 4 ml/h. The temperature was 4°C. ○-○, enzyme activity; ●-●, absorbance, 280 nm.

Dehydrogenases had identical elution volumes (Fig. 3), which indicated a molecular weight for the insect enzyme of approximately 70,000. A subunit molecular weight for the *A. suspensa* enzyme of 35,000 was determined by SDS gel electrophoresis (Fig. 4). The amino acid composition of the insect enzyme was determined by standard procedures and is compared in Table 2 with the composition of α-glycerophosphate dehydrogenases from a variety of other sources. The results of a pH optimum experiment with pure *A. suspensa* α-glycerophosphate dehydrogenase are shown in Figure 5. The enzyme shows maximal activity at pH 7 at 25°C.

**Immunology**

Initial experiments with the rabbit antiserum against *A. suspensa* α-glycerophosphate dehydrogenase showed that the serum strongly inhibited the enzymatic activity, which provided proof of the specificity of the antibody preparation.
A series of MC-F experiments were carried out using the purified A. suspensa α-GPDH and varying concentrations of the antiserum (Fig. 6A). A plot of the percentage complement fixed at the peak of fixation versus the logarithm of the antiserum dilution yielded a straight line, with a slope of 740 (Fig. 6B). MC-F were also done using as antigen crude tissue extracts of A. suspensa. These experiments were compared with those using purified antigen. The results of one such experiment are shown in Figure 7 and indicate that the two types of antigen preparation give equivalent results.

Titrations of heterologous antigens were made in essentially the same manner as for homologous antigens. Initial titrations established the appropriate dilution required to give 50–80% complement fixed. Once this range was established, closely spaced antiserum dilutions were used to determine the antiserum concentration required for 75% complement fixation. Representative results for both a closely related and a more distantly related antigen are shown in Figure 8. Since both pure homologous antigen and crude tissue extracts of this antigen gave identical titers, it was not necessary to use pure antigens for carrying our cross-reactions with heterologous antigens. Most of the heterologous antigens compared were in the form of crude tissue extracts, though in a few cases it was necessary to carry out an ammonium sulfate fractionation, followed by desalting, to reduce antigen complementarity of the antigen. From the results of the heterologous titration experiments, the degree of immunological relatedness of the heterologous antigens to A. suspensa L-glycerol-3-phosphate dehydrogenase was calculated in terms of immunity given in Table 3.

**DISCUSSION**

The procedure for phosphate dehydrogenase was developed for our most recent work and the purification procedure is given in Table 3. We have developed other tephritid α-GPDH by Kitto, unpublished work at the University of California, following gel filtration chromatography media were used: 5'-Monophosphate, Sepharose, and Tris-glycine. None proved as effective.

The physical characteristics of the enzymes are compared in Table 3.
in terms of immunological distance [2]. The results of these comparisons are given in Table 3 for a variety of tephritids and other Diptera.

**DISCUSSION**

The procedure described here for purification of *A. suspensa* α-glycerophosphate dehydrogenase has proven rapid and reliable. The overall yield for our most recent purifications is typically 50% or more of the initial activity, and the purification can be accomplished within 1 week. The slowest part of the procedure is the gel filtration step, in which it is necessary to use slow flow rates in order to maximize separation of the enzyme from other proteins. We have developed a general procedure for the purification of a variety of other tephritid α-glycerophosphate dehydrogenases (L. Davidson and G.B. Kito, unpublished data). In some of these cases it has been necessary to add an ion exchange chromatography step using diethylaminoethyl-cellulose following gel filtration to obtain pure enzyme. A variety of affinity chromatography media were tried for purification, including gels such as Adenosine-5′-Monophosphate-Sepharose, Trinitrobenzene-hexamethylenediamine-Sepharose, and Trinitrobenzene-adipic acid dihydrazide-Sepharose [29,30] but none proved as effective as the use of Affi-gel Blue.

The physical characteristics of the *A. suspensa* α-glycerophosphate dehydrogenase are comparable to those observed with homologous enzymes from
TABLE 2. Amino Acid Compositions of α-Glycerophosphate Dehydrogenases

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<td>334</td>
<td>318</td>
<td>325</td>
<td>328</td>
<td>315</td>
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* A: Anastrepha suspensa—this study; B: Drosophila melanogaster—Niesel [25]; C: Apis mellifera—Brosemer and Marquardt [41]; D: rabbit muscle—Van Eys et al. [49]; E: rat muscle—Fondy et al. [39]; F: chicken muscle—White [50].

All residues were normalized to a subunit molecular weight of 35,000.

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other sources. Gel filtration indicated an approximate molecular weight of 70,000 daltons for the A. suspensa enzyme, and the insect enzyme coeluted with rabbit muscle α-glycerophosphate dehydrogenase. A molecular weight of 70,000 daltons has been reported for the rabbit skeletal muscle enzyme by Ankel et al. [31]. Comparable molecular weights have been reported for several insect α-glycerophosphate dehydrogenases, including those from Ceratitis capitata [32], Drosophila melanogaster [33,34], Drosophila virilis [35], and

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Fig. 5. Effect of pH on the enzymatic activity of A. suspensa α-glycerophosphate dehydrogenase.

Fig. 6. An example of the effect of antisera concentration on the appearance of the antibody antigen peak and the antisera concentration as a percent of the control peak.
Fig. 6. An example of the titration of an antiserum (RS-AS-12) directed against purified *A. suspensa* α-glycerophosphate dehydrogenase. A: Complement fixation curves at differing antiserum concentrations. B: Relationship of the percent complement fixed at the reaction peak and the antiserum concentration.
Apis mellifera [36]. The subunit molecular weight of 35,000 estimated for the A. suspensa α-glycerophosphate dehydrogenase indicated that in the native form this enzyme is dimeric, as is the case with homologous enzymes from other species [25,32,33,37-40].

The A. suspensa α-glycerophosphate dehydrogenase has a pH optimum of 7 for the conversion of dihydroxyacetone phosphate to α-glycerophosphate at 25°C. This figure is similar to that found with several other adult insect α-glycerophosphate dehydrogenases [32,35,41-43] but differs substantially from the pH optimum at approximately 7.7 reported for rabbit skeletal muscle enzyme [41,44].

Our immunological studies using MCf indicate that α-glycerophosphate dehydrogenase is a very slowly evolving protein in the tephritids, as it is in hymenopteran species [10,12] and drosophilid species [11]. Within the limits of experimental error, our immunological results are in general agreement with traditional subfamily classifications of the Tephritidae based on morphology [45,46].

Electrophoretically distinct larval and adult isoensymes, which differ in tissue distribution and temporal expression, have been reported in insects [37,38,43]. Recent work has indicated that the two forms of the enzyme differ because of post-translational modification [47,48], but they could not be distinguished by double diffusion or immunoinactivation [25,49]. Using MCf, we have found that the larval and adult forms of tephritid α-glycerophosphate dehydrogenases show small but consistently repeatable differences in cross-reactivity. An example of this is shown in Table 3 for the enzymes from A. suspensa. Our immunological studies, however, revealed dehydrogenases than in the case of the A. suspensa enzyme.

Of particular interest, the amino acid sequence of the MCf enzyme from A. suspensa is identical to the amino acid sequence of the enzyme from A. mellifera [36]. This result provides strong evidence that the A. suspensa enzyme is indeed homologous to the A. mellifera enzyme. These results are consistent with the hypothesis that the A. suspensa enzyme is a homologous enzyme.

In addition, we have found that the MCf enzyme from A. suspensa is more similar to the MCf enzyme from A. mellifera than to the MCf enzyme from Drosophila melanogaster, which suggests that the A. suspensa enzyme is more closely related to the A. mellifera enzyme than to the D. melanogaster enzyme.

Fig. 7. A comparison of the complement fixation curves of purified A. suspensa α-glycerophosphate dehydrogenase and a crude enzyme extract from this species. The antiserum dilutions (RS-AS-12) are indicated on the figure.
enzymes from *Paranatha cultura*. The adult form of the *P. cultura* enzyme is immunologically closer to the *A. suspensa* adult α-glycerophosphate dehydrogenases than is the larval form.

Of particular interest from our studies is the finding of a strong degree of immunological resemblance between the α-glycerophosphate dehydrogenases of *A. suspensa* and the papaya fruit fly *Toxotrypana curvicauda*. The reciprocal cross-reaction, using an antibody directed against *T. curvicauda* α-glycerophosphate dehydrogenase, supports this relationship (J. Lemburg, H. Abbot, L. Davidson, and G.B. Kitto, unpublished data). These flies are, at least superficially, morphologically very dissimilar and have classically been placed in different subfamilies: *Toxotrypana* in the Dacinae and *Anastrepha* in the Trypticinae [46]. This classification is primarily based on morphological features, with *Toxotrypana* lacking ventral bullae, setae, and narrow wings. *Anastrepha* has these features. *Toxotrypana*, as presently classified, is the only New World representative of the Dacinae. From the immunological data, we suspect that the present classification has resulted from morphological convergence. In order to test this possibility we are presently examining the cross-reactivity of antibodies against a variety of other tephritid α-glycerophosphate dehydrogenases, with the *T. curvicauda* and *A. suspensa* enzymes. In addition, we are comparing the amino acid compositions and tryptic peptide maps of these purified enzymes. If, as our present data suggest, *Toxotrypana* belongs in the Trypticinae, then there are no Dacinae in the New World, and this latter subfamily must have arisen after connections between
<table>
<thead>
<tr>
<th>Species</th>
<th>Tribe</th>
<th>Immunological distancea</th>
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<tbody>
<tr>
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*Immunological comparisons were made using microcomplement fixation [1].


