It is of interest to note that the only electrophoretic analysis of insect peroxidases reported to date (Coles 1966) isolated three major PO isozymes in locusts. The three Drosophila PO's observed in the present study may well be homologous to the three locust isozymes. Electrophoretic analyses of the Drosophila peroxidases are currently in progress in our lab.

References: Armstrong,D., R.Rinehart, L.Dixon & D.Reigh 1978, Age 1:8-12; Aurand,L.W., W.M.Roberts & J.T.Cardwell 1956, J.Dairy Sci. 39:568-573; Coles,G.C. 1966, J.Insect Physiol.. 12:679-691; IUPAC-IUB Commission on Biochemical Nomenclature 1976, Handbook of Biochemistry and Molecular Biology, 3rd ed., V2, G.D.Fosman ed. CRC Press, Cleveland Ohio 1976 pp 84-84.

Poole, J.H. and L.K.Dixon. University of Colorado at Denver, Colorado USNA. Drosophila peroxidases: II. Isozyme kinetics, and optimum conditions for assays utilizing p-phenylenediamine.

The purpose of this study was to obtain initial estimates of the kinetic properties of Drosophila peroxidase (PO) isozymes, and to develop a sensitive photometric assay for each PO, for use on Drosophila tissue homogenates. A series of reactions was carried out to measure the effect of substrate concentration on reaction velocity, and to identify optimum assay conditions.

PROCEDURES: Flies were killed and extract prepared as described in the previous study in this series (see previous note). Subsequent to finding three major pH optima for PO activity (previous study), all extractions were carried out in tris-phosphate buffer (33.3 mM each) at pH 5.90, 7.50 or 9.05 (±0.01), 0.100 ml buffer/mg tissue. This yielded homogenates with the target pH of 5.9, 7.4 or 8.9 (±0.05).

PO activity was determined from the rate of oxidation of p-phenylenediamine (PDA) by hydrogen peroxide, measured as the change in absorbance at 485 nm (see previous note for general procedure). For each of the extracts (pH 5.9, 7.4, 8.9) a series of reactions was carried out with final concentration of H_2O_2 fixed at 20 mM, and the final concentration of PDA varied between 0.156 mM and 80 mM. This permitted estimation of the K_m for the PDA substrate. Following this determination, a series of reactions was carried out with the concentration of PDA fixed at 40 mM (i.e., greater than 75% enzyme saturation) and H_2O_2 concentration varied between 0.156 and 80 mM. This allowed estimation of the K_m for the H_2O_2 substrate.

KINETIC PROPERTIES OF THE PEROXIDASE ISOZYMES: Each of the three PO's produced linear Eadie-Hofstee plots (i.e., Michaelian kinetics) at all concentrations of PDA, and at concentrations of H_2O_2 below 5 mM. Catalatic activity was apparent as bubbling in the cuvet above 5 mM H_2O_2 --consistent with the observation of Angermueller and Fahimi (1981) that catalase activity predominates above 10 mM H_2O_2. The following estimates of K_m were obtained for each isozyme, based on the Eadie-Hofstee plots. For the H_2O_2 substrate, acid-PO had the highest apparent K_m=31±16.3 uM. Alkaline-PO's apparent K_m was 110±65 uM. For the PDA substrate, apparent K_m= 9±4.2 mM for acid-PO, 3.0±1.10 mM for neutral-PO, and 5.5±2.15 mM

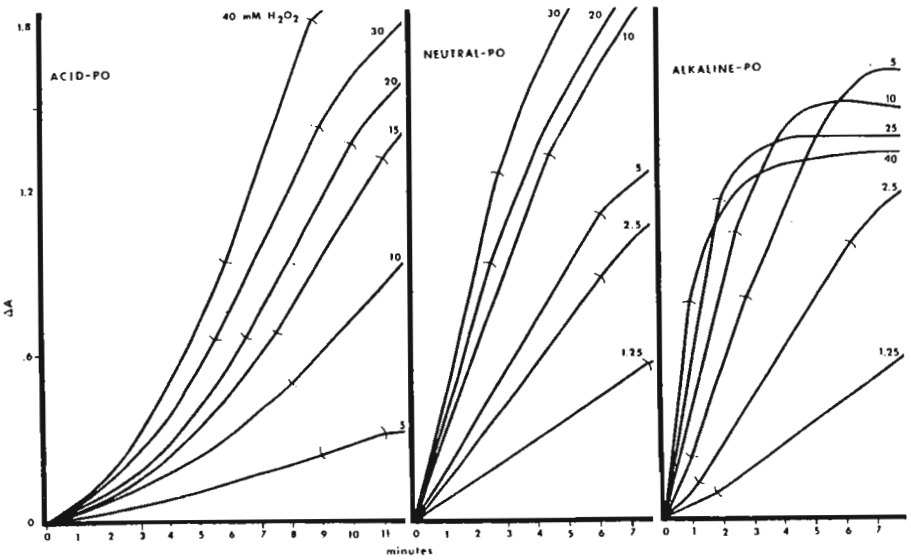

Fig. 1. Time course of the peroxidase reactions at various substrate concentrations. In all reactions the concentration of PDA was twice the concentration indicated for H_2O_2. Hysteretic lags were evident for acid-PO and alkaline-PO activity. Parentheses enclose the period of maximum linear velocity.

for alkaline-PO. It may be of interest to note that values of K_m are often indicative of the substrate concentrations normally encountered by an enzyme under physiological conditions (Hochachka & Somero 1973).

Two of the isozymes, acid-PO and alkaline-PO, displayed hysteretic kinetics (i.e, a lag phase prior to maximum linear reaction rate--Fig. 1). Alkaline-PO typically had a lag of 0-3 min before maximum velocity was attained. The lag for acid-PO was longer; an incubation period of 2-8 min was observed. For alkaline-PO the rate of transition to maximum velocity was found to be directly proportional to the concentration of PDA (r=0.991) and independent of changes in H_2O_2 concentration (Fig. 2). F r acid-PO the opposite was observed, with H_2O_2 concentration directly determining the rate of transition to maximum velocity (r=0.998). Frieden (1979) has pointed out that such hysteretic lags allow an enzyme to control substrate flux through metabolic pathways by damping the response to brief fluctuations in substrate concentration. This behavior may be important where a relatively constant level of enzyme activity is critical for homeostasis. In contrast, no hysteresis was observed for neutral-PO, which attained maximum linear velocity at the moment of substrate addition (Fig. 1).

OPTIMUM ASSAY CONDITIONS: In general a 1:2 ratio of H_2O_2 to PDA concentration was found to give best results. Raising the concentration of H_2O_2 above this ratio tended to produce bubbling in the cuvet, a catalatic reaction, which interfered with photon absorbance measurement. Lower levels of H_2O_2 reduced the rate and duration of linear reaction. Thus the 1:2 ratio produced the most rapid and sustained linear reaction.

A series of reactions was then carried out at the 1:2 ratio of substrates (ranging from 80 mM PDA + 40 mM H_2O_2 to 5.0 mM PDA + 2.5 mM H_2O_2). In this manner, the substrate conditions producing the most sustained linear reactions were obtained (Table 1).

STABILITY OF PEROXIDASE ACTIVITY IN HOMOGENATE: The peroxidase activity in the extract supernatant was found to change gradually over an eight hour period at 20°C. The acid-PO activity increased linearly at a rate of 5.7% ± 1.50% per hour. Neutral-PO activity decreased

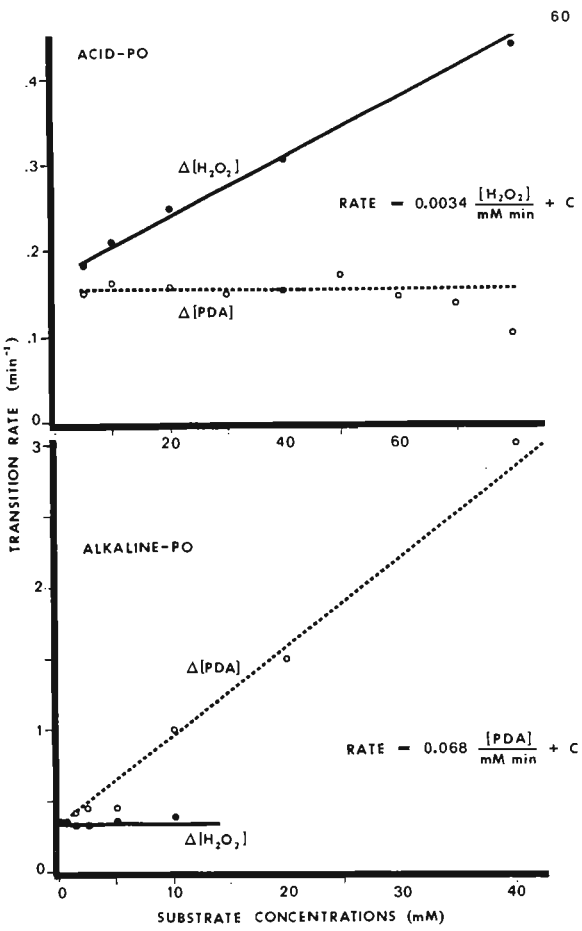

Table 1. Assay conditions for Drosophila peroxidase isozymes.

Volume	Initial Concentration	Final Concentration
ACID-PO ASSAY (pH 5.9)		
0.1 ml PDA	160.0 mM	20.0 mM
0.1 ml H_2O_2	80.0 mM	10.0 mM
0.6 ml extract	33.3 mM tris-phos	25.0 mM
0.8 ml total		
NEUTRAL-PO ASSAY (pH 7.4)		
0.1 ml PDS	20.0 mM	2.50 mM
0.1 ml H_2O_2	10.0 mM	1.25 mM
0.6 ml extract	33.3 mM tris-phos	25.00 mM
0.8 ml total		
ALKALINE-PO ASSAY (pH 8.9)		
0.1 ml PDA	40.0 mM	5.00 mM
0.1 ml H_2O_2	20.0 mM	2.50 mM
0.6 ml extract	33.3 mM tris-phos	25.00 mM
0.8 ml total		

For each assay, blanks were prepared as above, with plain buffer (pH 5.9, 7.4, 8.9) rather than extract.

Fig. 2. Effect of substrate concentration on the rate of hysteretic transition. The transition rate of acid-PO was dependent on H_2O_2 concentration, while that of alkaline-PO was dependent on PDA concentration.

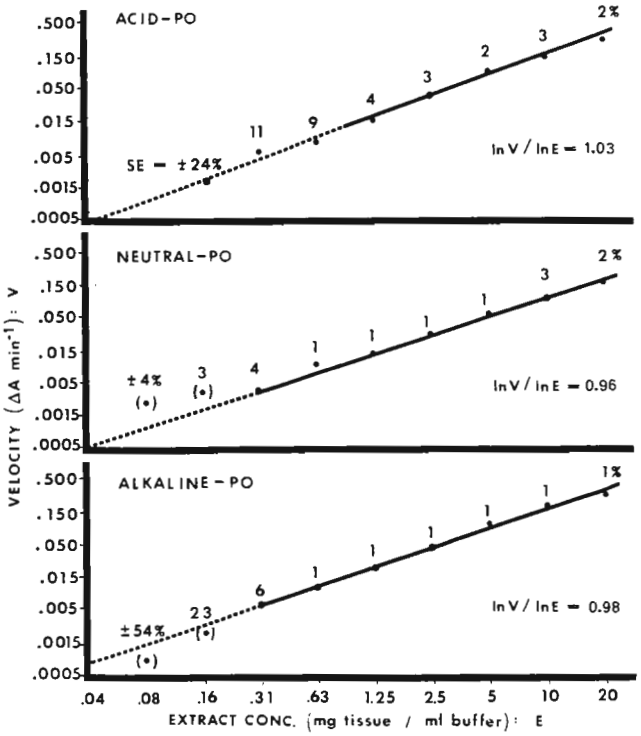

Fig. 3. Linearity and reproducibility of assays. Reaction rate vs extract concentration (by serial dilution) is graphed on a log-log plot. Relative standard error is indicated for each point (2 df). Solid lines indicate velocities that can be considered within the range of reliability for each assay (based on both linearity and reproducibility).

linearly at 2.7% ± 0.61% per hour. Alkaline-PO showed no significant change over time (0.0% ± 0.52% per hour). Consequently a standard period, post homogenization, was used for all assays.

STANDARD ASSAYS: Based on the above observations, the following protocol was established for assays of each isozyme's activity. After freezing, sexing and weighing, 5-10 organisms (or body segments) are placed in 1.00 ml buffer (33.3 mM tris-phosphate at pH 5.90, 7.50 or 9.05 (±0.01), prechilled to 3°C). The samples are homogenized with a motorized teflon glass tissue grinder, centrifuged (2400 rpm, 20 min) and the supernatant decanted for assay. Enzyme assays are then targeted to a one-hour window, set at 5.0 h (±0.5 h) after homogenization of samples.

See Table 1 for reagent proportions in each assay. Addition of extract initiates the reaction (time-zero), and A_{485} is recorded (±0.005) at intervals of 1 min (±5 sec), for 10 minutes.

The reaction velocity is calculated from the most linear portion of the curve over a minimum of three minutes (i.e., four readings). For acid-PO, these readings are usually at t=7 to 10 min: for neutral-PO and alkaline-PO at t=2 to 9 min.

SENSITIVITY AND REPRODUCIBILITY OF ASSAYS: Figure 3 summarizes findings on the usable range for each isozyme's assay, under the reaction conditions described above. Linearity of the assays, in response to various concentrations of PO is shown. Reproducibility is indicated by the value of the standard error at each level of activity (2 df).

The acid-PO assay was found to be linearly proportional to enzyme concentration at all levels of activity tested. On a log-plot plot, the slope is very close to unity (the index of direct proportionality), with a value of 1.03 (r=.997). The relative standard error is less than 5% for enzyme activities obtained with extract concentrations as low as 1.25 mg tissue/ml. This is equivalent to one fly per assay, under the above assay protocol. Reproducibility is marginal (SE>10%) at lower concentrations, equivalent to fractions of a fly per assay.

The neutral-PO and alkaline-PO assays were found to be three to five times as sensitive as the acid-PO assay. Log-log slopes of 0.96 and 0.98 (r=.999), as well as relative standard errors of less than 5%, were obtained for extract concentrations as low as 0.31 mg tissue/ml--or approximately one-third of a fly per assay, under the above assay protocol.

SCALING OF ASSAYS: The IUPAC-IUB Commission of Biochemical Nomenclature (1973) recommends use of katal units in all reports of enzyme activity (1 kat= the activity converting one mole substrate to product per second). It is recommended that use of the former "enzyme unit" (1 U = micromoles per minute) be discontinued.

In order to convert ΔA to katal units, the change in molar absorptivity (ε) at 485 nm, upon oxidation of PDA, was determined at each of the three assay pH's (based on absorbance measurements on standard solutions of PDA and oxidized PDA). At pH 5.9, $\Delta\varepsilon$=2.686±0.024. At pH 7.4, $\Delta\varepsilon$=2.137±0.0075. At pH 8.9, $\Delta\varepsilon$=1.98±0.078.

Based on the molar absorptivities, the extraction and assay volumes, the substrate concentrations used in these assays, and the values of K_m for each isozyme, the following

conversion factors may be readily calculated: acid-PO = 1.37×10^4; neutral-PO = 1.20×10^4; alkaline-PO = 1.23×10^4. Multiplying raw data of the assays (ΔA_{485} min^{-1}) by the above conversion factors gives the V_{max} PO activity of the 1 ml extract, in picokatals (pkat).

CONCLUSIONS: This assay procedure utilizes total soluble extracts of Drosophila tissue, without prior concentration or isolation of the PO isozymes. Thus, the measurements of enzyme activity cannot be assumed to reflect enzyme concentration alone, but may also be influenced by natural inhibitors and activators of peroxidases, present within the tissue homogenates. The method has the advantage of speed and yields reasonably reproducible results. We are currently analyzing the PO isozymes of Drosophila for developmental changes in activity, tissue distribution and functional significance.

References: Angermueller,S. & H.D.Fahimi 1981, Histochemistry 71:33-44; Frieden,C. 1979, Ann.Rev.Biochem. 48:471-489; Hochachka,P.W. & G.N.Somero 1973, Strategies of Biochemical Adaption, W.B.Saunders Co., Philadelphia; IUPAC-IUB Commission on Biochemical Nomenclature 1976, Handbook of Biochemistry and Molecular Biology, 3rd ed, V2, G.D.Fosman ed., CRC Press, Cleveland, Ohio 1976, pp.84-87.

Poole, J.H. and L.K.Dixon. University of Colorado at Denver, Colorado USNA. Drosophila peroxidases: III. Developmental profile of isozymes.

In this report, we present our findings on the expression of peroxidase (PO) isozyme activity over the lifespan of Drosophila melanogaster.

METHODS: Wildtype stocks were maintained on cornmeal-molasses-agar-yeast medium in half-pint bottles, with a diurnal cycle of 12 hr in the light at 30°C and 12 hr in the dark at 20°C. Imagoes were collected at eclosure and transferred weekly to fresh medium, thereby maintaining distinct age-cohorts. For assay, third instar larvae were collected while wandering outside the medium prior to pre-pupal immobilization. Pupae were collected after completion of body segmentation and wing-bud/leg eversion but prior to initiation of eye pigmentation. Imagoes were collected for assay at eclosure ("week 0") and at weekly intervals thereafter. For each assay 6-10 organisms of a given age and sex were frozen, weighed, homogenized in buffer, centrifuged and photomerically assayed with PDA/H_2O_2 as previously described (see Report II). In addition, protein content of the supernatant was assayed by UV absorbance at 260 and 280 nm (Layne 1957). Three independent extractions and assays were performed for each PO isozyme, at each age and sex (except for the last two weeks of life, when sufficient numbers of organisms survived to perform only 2 independent determinations). Larvae and pupae were not sexed for this series of assays. PO activity is reported in picokatals (picomoles H_2O_2 reduced per second) at V_{max}, per mg body mass (see previous note for conversion factors).

PO ISOZYME ACTIVITIES DURING DEVELOPMENT: The activity of each PO isozyme across the lifespan of wildtype D. melanogaster is shown in Figure 1. Each data point represents the mean and standard error of the mean for PO activity at a given age.

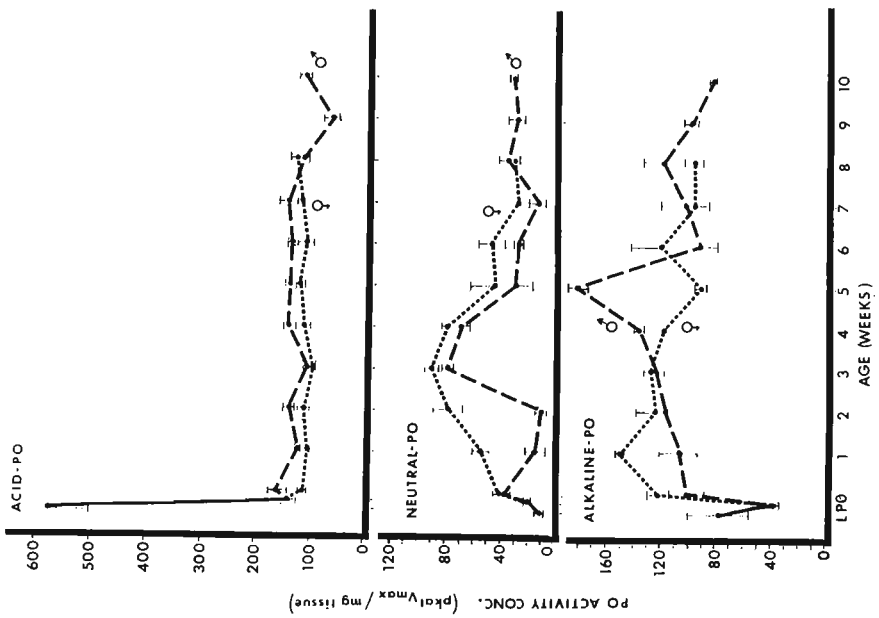

Fig. 1. Activity concentration of peroxidase isozymes vs age (wildtype females and males).