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Microsomal epoxidase activity in preparations of whole southern armyworm larvae

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INSECT microsomal oxidases are important in the metabolism of insecticides and other foreign compounds. Earlier studies of the microsomal oxidases in the southern armyworm (*Prodenia eridania* Cramer)[1] and other lepidopterous larvae[2] indicated that both whole larvae homogenates and microsomes were without detectable activity. Active oxidase systems were obtained from isolated tissues, including gut, fat body and Malpighian tubules, and in the armyworm midgut provided an extremely active epoxidase preparation.[1] The inactivity of the whole larvae preparations is due in part, if not wholly, to an endogenous inhibitor in the gut contents. This material has been partially purified and characterized as a serine proteinase with a molecular weight of 26,000.[3] Kahn[4] and Kuhr[5] have independently confirmed the observation that whole caterpillar homogenates are not suitable sources of microsomal oxidase.

Therefore, the recent report of Williamson and Schechter[6] concerning microsomal aldrin epoxidation in preparations of whole larvae was of considerable interest. They obtained active microsomal epoxidase systems from whole larvae homogenates of five species of caterpillars. These workers observed that the darkening reaction catalyzed by the phenolase complex was associated with reduced epoxidase activity and that epoxidase activity was enhanced by the addition of bovine serum albumin and 2-phenylthiourea to the incubation media. It is also noteworthy that whole larvae preparations have been used by Fukami and Shishido[7] in studies of phosphorothioate metabolism in the rice stem borer, but the biochemical properties of the preparations have not been reported in detail.

This report is a reconciliation of some of the findings of Williamson and Schechter[6] with earlier observations concerning the suitability of whole larvae homogenates for studies of microsomal oxidases in Lepidoptera.

Insects. Southern armyworm, *Prodenia eridania* Cramer, larvae were reared in the greenhouse on red kidney bean leaves as previously described.[1] Actively feeding sixth-instar larvae, burrowing larvae, prepupae and pupae were used. "Burrowing larvae" designates larvae with cleared digestive tracts that were burrowing in the sand prior to pupation. "Prepupae" refers to larvae which were shortened and were essentially immobile.

Chemicals. Analytical grade aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo, exo-5,8-dimethanonaphthalene) and its 6,7-epoxide, dieldrin, were supplied by the Shell Development Company, Modesto, Calif. Glucose 6-phosphate (G6-P), G6-P dehydrogenase and NADP were purchased from Calbiochem, Los Angeles, Calif. All other chemicals and solvents were analytical reagent grades.

Enzyme preparation. The larvae or pupae (approximately 3 g) were ground in an ice-cold mortar and pestle which contained 8 ml of 67 mM sodium-potassium phosphate buffer, pH 7·8. The brei plus 8 ml buffer was transferred to a glass-Teflon tissue grinder and was mechanically homogenized until no large debris remained. The final homogenate (16 ml) was 15–20% (w/v), and an 8-ml aliquot was centrifuged in an International Equipment Company preparative ultracentrifuge (model B-60, rotor 494). A preliminary centrifugation (12,000 *g* for 10 min) provided the supernatant from which the microsomal fraction was sedimented by centrifugation at 110,000 *g* for 60 min. The resulting supernatant is designated the "soluble" fraction. Protein was estimated by the biuret method of Fincham[8] using bovine serum albumin standards.

Incubation procedure and epoxidase activity. The standard 5-ml incubation medium contained the following (final concentrations):[1] tris–HCl buffer, pH 7·8 ($5{\cdot}0 \times 10^{-2}$M); G6-P ($2{\cdot}4 \times 10^{-3}$M); G6-P dehydrogenase (2 units); NADP ($5{\cdot}1 \times 10^{-5}$M); KCl ($2{\cdot}7 \times 10^{-3}$M); and aldrin (100 μg added in 25 μl ethanol). The reaction was initiated by enzyme addition and the mixture was shaken in a water bath at 30° during the 10-min incubation period. Epoxidation was terminated by acetone addition and the chlorohydrocarbons were extracted into hexane. The extracts were analyzed for dieldrin by electron-capture gas chromatography as previously detailed.[1]

Microsomal epoxidase activity is expressed as the number of nanomoles of dieldrin formed per milligram of protein per minute.

Effect of developmental condition of larvae on microsomal epoxidase activity. Earlier studies had shown that homogenates of whole feeding larvae were without detectable epoxidase activity and that the larval gut was the most satisfactory enzyme source in the armyworm[1] and other lepidopterous

larvae.[1,2] Comparative studies demonstrated that maximum activity was found in gut homogenates of feeding larvae in the last feeding stage (instar) and that activity dropped sharply after the feeding period. Williamson and Schechter[6] obtained active preparations from homogenates of "late instar larvae prior to pupation", but the effect of larval age was not considered. In the present study it was postulated that larval age might be of prime importance in enabling the preparation of active oxidases from homogenates of whole larvae.

Following the procedures of Williamson and Schechter,[6] microsomal fractions were prepared from whole armyworm larvae in several different developmental conditions. As previously reported, whole feeding larvae homogenates yielded microsomes without detectable epoxidase activity (Table 1). However, homogenates of armyworms which had ceased feeding and were in the burrowing, prepupal, and pupal conditions yielded active microsomes. Lower levels of epoxidase activity were found in each succeeding developmental stage. A similar observation had been made when epoxidase activity was measured in armyworm gut homogenates.[1,2] It is not clear at this time whether the different levels of activity are due to different amounts of enzyme, of endogenous inhibitors or of both.

TABLE 1. EPOXIDASE ACTIVITY OF MICROSOMAL FRACTIONS OF SIXTH-INSTAR ARMYWORMS IN DIFFERENT STAGES OF DEVELOPMENT*

Enzyme source	N	Epoxidase activity (nmoles dieldrin/mg protein/min)
Whole feeding larvae	2	none detectable
Feeding larvae (−gut contents)	4	1·44 ± 0·28
Burrowing larvae	7	0·21 ± 0·12
Prepupae	7	0·07 ± 0·04
Pupae	10	0·04 ± 0·01

* The microsomal fraction was obtained from whole insect homogenates prepared in 67 mM Na-K phosphate buffer (pH 7·8). Standard incubation procedure was employed and the incubation mixtures contained 1–5 mg microsomal protein. Means ± S.D.; N = number of experiments.

Endogenous inhibitors in the food are probably responsible for the inactivity of the whole feeding larvae preparations.[3] This view is supported by the observation that active microsomal preparations were obtained from whole feeding larvae from which the food bolus had been removed by dissection before homogenization. The specific activity of these preparations (1·44 units, Table 1) is markedly lower than that which has been obtained using armyworm midgut microsomes (1·8–4·5 units).[2] This observation provides a direct demonstration of the tissue dilution factor[1] incurred when the enzyme source is a homogenate of whole insects rather than a brei of a specific tissue or organ system.

Effect of the darkening reaction on microsomal epoxidase activity. When Williamson and Schecter[6] employed the mitochondrial supernatant (soluble + microsomes) as their epoxidase source, they observed that the darkening reaction catalyzed by the phenolase complex[9,10] seriously interfered with the determination of epoxidase activity. The soluble phenolase system is important in the tanning of insect cuticle[9] and is similar to the microsomal epoxidase system in its requirement for NADPH and oxygen. They observed up to 3·8-fold stimulation of epoxidase activity when phenylthiourea (PTU), a phenolase inhibitor, and bovine serum albumin (BSA) were added to the incubation media. Since BSA stimulation occurred both in the presence and absence of the soluble fraction, the effect is probably not related to the darkening phenomenon.

Williamson and Schechter[6] postulated that PTU enhanced epoxidase activity by blocking the darkening reaction, thereby making more NADPH and oxygen available to the microsomal epoxidase system. In the present study, experiments were conducted to assess whether there was competition for NADPH and oxygen between the darkening reaction and epoxidation or whether the products of the reaction were inhibitory. Microsomal supernatant was the source of phenolase used and the results of the experiments are shown in Table 2.

When microsomal supernatant was added before any darkening was detectable, control levels of activity were measured and no stimulation of epoxidation was obtained by inclusion of PTU (1 mM) in the media. In other cases, the soluble fraction was darkened by bubbling air through it for 5 min and, when small portions of the darkened supernatant were added to standard epoxidase media, the level of epoxidase activity was sharply reduced (Table 2). Neither PTU nor BSA reduced the inhibition which occurred in the presence of darkened supernatant. These results suggest that the

inhibition of epoxidation by the soluble fraction is due to the products of the darkening reaction rather than to the reaction *per se*.

TABLE 2. EFFECT OF ADDITION OF SOLUBLE FRACTION OF BURROWING LARVAE HOMOGENATE TO EPOXIDASE ASSAY SYSTEM*

Addition	Epoxidase activity (%)
None	100
Supernatant	105
Darkened supernatant†	66
Supernatant + PTU (1 mM)	103
Darkened supernatant + PTU‡	70
Darkened supernatant + BSA (5 mg/incubation)	65

* Homogenate of whole burrowing larvae was prepared (1 larva/2 ml) in 67 mM Na-K phosphate buffer (pH 7·8). The complete system was as detailed in Table 1. Means of two experiments. PTU = phenylthiourea; BSA = bovine serum albumin.

† Supernatant was darkened by bubbling with air for 5 min at 30° before addition to incubation medium.

‡ PTU was added after darkening by bubbling with air.

In the course of the larval age–epoxidase activity experiments, marked differences in the rates and extent of darkening in the various preparations were noted. These parameters were assessed to determine whether there was a positive correlation between the degree of darkening of the soluble fraction and the epoxidase activity of the corresponding microsomes. If the products of the darkening reaction alone were responsible for the dramatic decrease in epoxidase activity observed after the feeding period (Table 1), it would be expected that the extent of darkening would be progressively greater in burrowing larval, prepupal and pupal preparations. The results in Table 3 indicate that the burrowing larval and prepupal preparations darken most readily and to the greatest extent. The pupal preparations darkened less readily and to a lesser extent than either of the above and, since the pupal microsomes had the lowest epoxidase activity, the darkening reaction does not seem to be the causal factor.

TABLE 3. DARKENING OF SOLUBLE FRACTION OF HOMOGENATES OF SIXTH-INSTAR ARMYWORMS AND PUPAE*

Soluble fraction	Degree of darkening		
	15 min	30 min	90 min
Feeding larvae minus gut contents	ND	ND	+
Burrowing larvae	++	+++	+++
Prepupae	++	+++	+++
Pupae	+	++	++

* The soluble fractions were decanted without delay and held in open Erlenmeyer flasks in a water bath at 30°. The degree of darkening was estimated by comparing the preparations to a "completely" darkened one obtained from burrowing larvae by bubbling it for 5 min with air. ND, none detectable; +, slight; ++, moderate; +++, complete.

In future biochemical investigations of factors affecting the levels of oxidase activity in larvae after the cessation of feeding, it may be useful to monitor phenolase activity in the haemolymph in order to obtain larvae in carefully defined physiological conditions.

In conclusion, larval age is of paramount importance in enabling the preparation of active microsomal oxidases from homogenates of whole armyworm larvae. Endogenous inhibitory activity in the gut contents prohibits the use of homogenates of whole feeding larvae but, after the food bolus has been voided prior to pupation, whole larval homogenates yield active microsomal fractions.

Progressively lower levels of activity are obtained in burrowing larval, prepupal and pupal preparations. Feeding larvae are more suitable than late instar larvae as sources of microsomal oxidases, since they yield preparations with higher specific activity and, additionally, the levels of larval oxidase are relatively stable during the feeding period.[2]

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Regional distribution of persistently bound reserpine in rat brain*

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RECENT reports from this laboratory dealing with the specific binding of reserpine have focused on binding of the drug in peripheral organs.[1–3] The present communication describes the regional distribution of reserpine in rat brain after intravenous administration. Evidence is presented for the specificity of persistent reserpine binding in brain.

Female Sprague–Dawley rats, 160–180 g, received intravenously 200 μg/kg of [^{3}H]reserpine (New England Nuclear Corp., 424 mc/m-mole, labeled in the trimethoxybenzoic acid moiety). The rats were killed by chloroform asphyxiation 6, 18 or 42 hr after drug administration. Brains were removed and dissected into seven regions as described by Glowinski and Iversen,[4] the regions being cerebellum, medulla-pons, striatum, midbrain, hypothalamus, cerebral cortex, and hippocampus. The concentration of [^{3}H]reserpine was measured in each region as described previously.[1]

Concentrations of [^{3}H]reserpine in certain parts of rat brain such as striatum and midbrain were quite variable. As shown in Table 1, the lowest [^{3}H]reserpine concentrations were found in the cerebellum while the highest concentrations were found in the striatum. Levels in the striatum at 18 hr, however, did not differ significantly from those in the medulla, and levels in the cortex did not differ significantly from those in the midbrain. There was relatively little change in the observed concentrations in the various regions between 6 and 42 hr.

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