Properties of 5-Aminolaevulinate Synthetase and its Relationship to Microsomal Mixed-Function Oxidation in the Southern Armyworm (*Spodoptera eridania*)

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1. Activity of 5-aminolaevulinate synthetase was measured in the midgut and other tissues of the last larval instar of the southern armyworm (*Spodoptera eridania* Cramer, formerly *Prodenia eridania* Cramer). 2. Optimum conditions for measuring the activity were established with respect to all variables involved and considerable differences from those reported for mammalian enzyme preparations were found. 3. Maximum activity (20 nmol/h per mg of protein) occurs 18-24 h after the fifth moult and thereafter decreases to trace amounts as the larvae age and approach pupation. 4. Synthetase activity was rapidly induced by oral administration (in the diet) of pentamethylbenzene, phenobarbital, diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate, and 2-allyl-2-isopropylacetamide. 5. Puromycin inhibited the induction of synthetase by pentamethylbenzene. 6. Induction of 5-aminolaevulinate synthetase correlated well with the induction of microsomal *N*-demethylation of *p*-chloro-*N*-methylaniline, except for phenobarbital, which induced the microsomal oxidase relatively more than the synthetase.

The initial precursor of the haem component of microsomal cytochrome *P*-450 (and other cytochromes) is the 5-aminolaevulinate produced in mitochondria from glycine and succinyl (3-carboxypropionyl)-CoA by the enzyme 5-aminolaevulinate synthetase. This enzyme is considered to be involved in the rate-limiting step in haem biosynthesis and consequently may play a critical regulatory role in the formation of cytochrome *P*-450 (Granick & Urata, 1963). The enzyme is induced by a multitude of xenobiotics known to induce the microsomal mixed-function oxidases (Baron & Tephly, 1970), and a correlation has been shown between the amount of 5-aminolaevulinate induction and microsomal mixed-function oxidase activity (Marver, 1969).

Recent studies have established that cytochrome *P*-450 and mixed-function oxidase activity in the larval gut tissues of the southern armyworm (*Spodoptera eridania* Cramer, formerly *Prodenia eridania* Cramer) are highly susceptible to induction by a variety of compounds, notably the methylated benzenes (Brattsten & Wilkinson, 1973). The remarkably rapid response of these enzymes to the presence of an inducing agent in the diet suggests that the armyworm might make a useful model for studies on the mechanism of induction and the regulation of microsomal mixed-function oxidase activity.

In view of the potentially important regulatory role of 5-aminolaevulinate synthetase in the formation of cytochrome *P*-450, studies were initiated to charac-

terize this enzyme in the gut tissues of southern armyworm larvae. Only one previous report (Chan *et al.*, 1973) on 5-aminolaevulinate synthetase in insect tissues, in relation to the cytochrome *c* content in tobacco-hornworm (*Manduca sexta*) flight muscle, is available.

Experimental

Materials

Chemicals. Glycine, *p*-chloro-*N*-methylaniline, *p*-chloroaniline, NADP⁺, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and 5-aminolaevulinate (hydrochloride salt, B-grade), were purchased from Calbiochem, La Jolla, Calif., U.S.A. *S*-Succinyl-CoA, pyridoxal phosphate, cytochrome *c*, L-ascorbic acid, puromycin (dihydrochloride salt, crystalline form), and porphobilinogen were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Pentamethylbenzene and *p*-dimethylaminobenzaldehyde were from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Aminoacetone and 2-allyl-2-isopropylacetamide were from K & K Laboratories, Plainview, N.Y., U.S.A. Diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate was purchased from Aldrich Chemical Co., Milwaukee, Wis., U.S.A., and sodium phenobarbital was from Merck and Co., Rahway, N.J., U.S.A. Samples of 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 provided by Shell Development Co., Modesto, Calif., U.S.A. [^{14}C]Glycine (114mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals and solvents used were of reagent-grade quality.

Insects. A colony of the southern armyworm (*Spodoptera eridania* Cramer) was maintained as described by Krieger & Wilkinson (1969). Last (sixth)-instar larvae were kept in a constant-climate room (21°C, 25% relative humidity, 24h light) before use.

Methods

Enzyme preparation. Midguts from groups of 15–30 larvae, depending on age (size) in the last instar, were opened by longitudinal section and cleaned and homogenized in 0.9% NaCl in a ground-glass tube with a motor-driven Teflon pestle at moderate speed for 30s. This crude homogenate was centrifuged for 3min at 1000g in a chilled centrifuge. The pellet was rehomogenized in 0.9% NaCl and re-centrifuged as above. The combined supernatants (S_1) were then centrifuged for 10min at 13000g (r_{av} 7.5cm) at 2°C in the IEC model B-20A centrifuge with a fixed-angle head (874) and the pellet (P_2) resuspended in 0.9% NaCl was used for measurements of 5-aminolaevulinic synthetase activity.

The microsomal pellets (P_3) obtained by centrifugation of a postmitochondrial supernatant for 1h at 100000g (r_{av} 4.5cm) at 2°C in an IEC model B-60 preparative ultracentrifuge and resuspended in 1.15% (w/v) KCl or a crude homogenate of midguts in 1.15% KCl as described in Brattsten & Wilkinson (1973) were used for measurements of aldrin epoxidation and the *N*-demethylation of *p*-chloro-*N*-methylaniline.

Enzyme assays. Optimum conditions for measurement of 5-aminolaevulinic synthetase activity were developed by modification of several methods described for mammalian hepatic preparations (Tschudy *et al.*, 1964; Wada *et al.*, 1968; Scholnick *et al.*, 1972; Whiting & Elliott, 1972; Hayashi *et al.*, 1972). The effects of several parameters on 5-aminolaevulinic synthetase activity are reported in this paper. The incubation mixture finally adopted for optimum synthetase measurements consisted of 180μmol of sodium phosphate buffer, pH 7.2, 100μmol of glycine, 0.80μmol of EDTA, 0.50μmol of pyridoxal phosphate, 0.58μmol of succinyl-CoA and 0.4–0.7mg of protein in a total volume of 3.0ml. The reactions were left to proceed aerobically for 30min at 40°C in a Lab-Line Instruments metabolic incubator and were terminated by the addition of 2.0ml of cold (15°C) 10% (w/v) trichloroacetic acid. After deproteinization, 0.1ml of 2,4-pentanedione was added to 3.0ml of the acid supernatant and the pH was adjusted to 5.6 with 0.9ml of 1M-sodium acetate buffer. This mixture was held at 100°C in a Scientific Products Tempblock Module heater for

25min, and after cooling, 3.0ml was mixed with an equal volume of modified Ehrlich's mercury reagent (Urata & Granick, 1963). After 15min at room temperature (21°C) the extinction of the samples was read at 552nm against zero-time blanks in a Unicam SP.800A u.v. spectrophotometer equipped with scale-expansion accessories. A sample containing standard 5-aminolaevulinic acid (converted into the pyrrole form), at a final concentration of 1.875nM, was included with each experiment. Porphobilinogen was measured by mixing 3.0ml of the acid supernatant directly with an equal volume of the modified Ehrlich's mercury reagent and reading the extinction against zero-time blanks at 552nm. In the incubation with [^{14}C]glycine, 60μmol of unlabelled glycine and 0.5μCi of [^{14}C]glycine were used instead of the 100μmol used as a routine. After conversion into the pyrrole form as described above the volume of the sample was decreased by elution with methanol–acetic acid (2:1, v/v) from a column (2cm × 1cm) of Dowex 1 anion exchange resin (X8; 200–400 mesh) converted into the acetate form with sodium acetate (0.5–2M). The eluate was evaporated under a stream of dry N_2 , redissolved in methanol and applied to a t.l.c. plate (precoated, 0.25mm thick, silica gel, 60F-254; EM Reagents, Darmstadt, Germany). The plate was developed in a solvent system of methyl acetate–propan-2-ol–7.1M- NH_3 (45:35:20, by vol.) as described by Irving & Elliott (1969) and analysed in a Packard Radiochromatogram Scanner model 7201.

The Ehrlich-positive products in the incubation mixture were separated by column chromatography and t.l.c. as above by using incubations with unlabelled glycine. Standards of 5-aminolaevulinic acid and aminoacetone were co-chromatographed with samples after pyrrole conversion, and a standard of authentic porphobilinogen was co-chromatographed with the samples. In this case the chromatograms were sprayed with diluted aq. Ehrlich's mercury reagent (1:1, v/v) for detection of the products.

Cytochrome *c*-oxidase activity was measured with a YS1 4004 Clark oxygen probe connected to a Gilson Oxygraph KM at 30°C in an incubation system containing 140μmol of sodium phosphate buffer, pH 7.2, 81μmol of cytochrome *c*, 14μmol of L-ascorbic acid and 0.1mg of protein in a total volume of 1.5ml. The results are expressed as percentage of O_2 uptake/min per mg of protein. The method was modified from that of Okamoto *et al.* (1967).

Epoxidation of aldrin was measured as described by Krieger & Wilkinson (1969) and the *N*-demethylation of *p*-chloro-*N*-methylaniline was as described by Krieger & Wilkinson (1970).

Protein concentrations were determined by a modified biuret method (Fincham, 1954), with bovine serum albumin as standard.

All incubations were in duplicate and all experiments were repeated three or four times.

Administration of chemicals. Armyworm larvae, all of the same age (± 1.5 h) were maintained on a semi-defined artificial diet (Brattsten & Wilkinson, 1973) into which the test compound was incorporated in known amounts. A second group of larvae of exactly the same age was fed on control diet *ad libitum*. Enzyme activities were assayed simultaneously in preparations from control and treated larvae.

Results

Incubation conditions

Optimum conditions were established for the measurement of 5-aminolaevalinate synthetase activity *in vitro* in the mitochondrial pellet (P_2) and the incubation mixture and conditions finally adopted are those described in the Experimental section.

The use of both Tris-HCl and sodium phosphate buffers in the pH range 5.8–7.8 resulted in optimum synthetase activity at pH 7.2 (Fig. 1a). The use of Tris-HCl buffer resulted in slightly higher enzyme activities at pH values below 7.4. The opti-

mum buffer concentration in the incubation mixture for enzyme activity was at about $180 \mu\text{mol}/3.0 \text{ ml}$, but apparently buffer concentration does not have a major influence on enzyme activity (Fig. 1b). In contrast, variation in the concentration of EDTA had a dramatic effect on activity, as shown in Fig. 1(c). Enzyme activity was optimum at EDTA concentrations within the range of 0.75 – $1.0 \mu\text{mol}/\text{incubation}$ and decreased sharply in incubation mixtures containing less than $0.5 \mu\text{mol}$ or more than $1.25 \mu\text{mol}$. The pyridoxal phosphate concentration was also critical, and gave maximum activity at $0.5 \mu\text{mol}/\text{incubation}$ (Fig. 1d). The addition of more than $100 \mu\text{mol}$ of glycine to the incubation mixture resulted in a decrease in specific activity. The K_m for *S*-succinyl-CoA was $2 \times 10^{-5} \text{ M}$ and addition of increasing amounts of this co-substrate caused only very small increases in specific activity.

The 5-aminolaevalinate synthetase activity was very sensitive to temperature as shown in Fig. 2. Optimum activity occurred at 40 – 42°C although a sharp decline was observed at higher temperatures.

Fig. 1. Incubation conditions for 5-aminolaevalinate synthetase

(a) Effect of pH. $\circ$, Tris-HCl buffer; $\bullet$, potassium phosphate buffer. (b) Effect of concentration of Tris-HCl buffer. (c) Effect of EDTA concentration. (d) Effect of pyridoxal phosphate concentration. The total incubation volume was 3.0 ml . Incubations proceeded aerobically for 30 min with gentle shaking. Full experimental details are given in the text.

Fig. 2. Effect of incubation temperature on 5-aminolaevulinate synthetase activity

Experimental details are given in the text.

Fig. 3. Effect of protein concentration on 5-aminolaevulinate synthetase activity

The mitochondrial pellet (P_2) was used in the incubations. Protein concentrations were determined by a modified biuret method with bovine serum albumin as standard.

At 40–42°C the reaction rate was linear with time up to 35 min.

Activity increased linearly with the amount of mitochondrial protein per incubation up to 1.5 mg per incubation but the reaction rate was suppressed at higher concentrations (Fig. 3). The specific activity data were most reproducible in the range of 0.4–0.7 mg of protein/incubation.

The mitochondrial preparation was normally used without delay for enzyme assays. Suspensions stored in ice for 18 and 40 h lost 50 and 75% of their 5-aminolaevulinate synthetase activities respectively.

Product measurement and identification

The recovery of 5-aminolaevulinate in the form of the pyrrole colour complex with *p*-dimethylaminobenzaldehyde was better than 90% in all experiments. This was checked by including with each experiment a sample containing denatured enzyme and a known amount of 5-aminolaevulinate and by using the maximum molar extinction coefficient of $61\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Fig. 4). Fig. 4 shows that a 25 min incubation at 100°C was necessary for maximum pyrrole formation under the conditions used; longer incubation periods at 100°C gave lower apparent extinction coefficients. T.l.c. examination of a standard sample containing added porphobilinogen showed that this pyrrole was destroyed by incubation in an acid medium (pH 5.6) at 100°C for 25 min.

With the use of standards, the elution pattern from the Dowex column was as shown in Fig. 5. On the t.l.c. plates the R_F values of [^{14}C]glycine and porphobilinogen were 0–0.10. The 5-aminolaevulinate-pyrrole migrated with the R_F value of 0.48–0.50, whereas the aminoacetone-pyrrole closely followed the solvent front (R_F value of 0.90–1.0). T.l.c. examination of the combined appropriate column fractions from the enzyme incubations showed the presence of 5-aminolaevulinate-pyrrole only after the plate had been sprayed with diluted Ehrlich's mercury reagent. This was confirmed by the results from incubations with [^{14}C]glycine.

Fig. 4. Apparent molar extinction coefficient (ϵ_{app}) for the colour complex formed by the 5-aminolaevulinate-pyrrole and the modified Ehrlich's mercury reagent

Two final concentrations of 5-aminolaevulinate (25 and 40 nM) gave identical results.

In the incubation system designed to measure synthetase activity, porphobilinogen, which could be formed as a result of 5-aminolaevulinate dehydrase activity in the preparations, was formed in appreciable amounts (1.10nmol/h per mg of protein) only after the addition of 200nmol of 5-aminolaevulinate. This concentration was never achieved under normal incubation conditions and the formation of porphobilinogen is consequently not considered to be a problem in the synthetase measurements.

Localization of 5-aminolaevulinate synthetase activity

5-Aminolaevulinate synthetase activity was present in all tissues of the southern armyworm that were examined. The midgut had the highest activity fol-

lowed in decreasing order by the fat-body and the Malpighian tubules. Only traces of activity were found in the foregut, hindgut and the silk gland. The S₁ supernatants from tissue homogenates of normal, 24h-old (±1.5h), sixth-instar larvae were used for the tissue-localization experiments.

Table 1 shows the distribution of 5-aminolaevulinate synthetase activity in several subcellular fractions of the armyworm midgut tissue. Cytochrome *c* oxidase and aldrin epoxidase activities were also measured as markers for mitochondria and microsomal preparations respectively. The data show that maximum synthetase activity as well as cytochrome *c* oxidase activity resides in the mitochondrial fraction (P₂) obtained by centrifugation of S₁ for 10min at 13000g_{av.}. Some synthetase activity was lost in the first pellet (P₁) containing mainly nuclei and cell debris, and a small portion remained in the mitochondrial supernatant (S₂). The mitochondrial pellet (P₂) also contains some of the aldrin epoxidase activity, although as expected this was concentrated mainly in the microsomal pellet (P₃).

Age variation and induction

Fig. 6 shows the variation in synthetase activity in the midguts of last-instar armyworm larvae during the period from the fifth larval moult to the time of pupation. Activity increased rapidly during the first 15h of the sixth instar by which time it had attained a maximum specific activity of about 20nmol/h per mg of protein. This activity was maintained for only a relatively short period and thereafter declined to only trace values in 4-day-old larvae. Addition of enzyme from 1-day-old larvae to preparations from larvae of 4 and 5 days of age showed that the variation was real and not due to the presence of endogenous inhibitory materials in the older larvae.

Fig. 6 also shows the effect on synthetase activity of continuously feeding pentamethylbenzene or diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-di-

Fig. 5. Elution of Ehrlich-positive compounds and glycine from a column (1 cm×2 cm) of Dowex 1 (X8)

Glass-distilled water (16ml) was added to the column and the fractions containing porphobilinogen and excess of glycine were collected. The fractions containing the pyrroles were collected by using methanol-acetic acid (2:1, v/v) as eluent. 5-Aminolaevulinate-pyrrole (—); aminoacetone-pyrrole (----); [¹⁴C]glycine (---); porphobilinogen (.....).

Table 1. Distribution of 5-aminolaevulinate synthetase, cytochrome *c* oxidase and aldrin epoxidase activities in subcellular fractions of midguts from normal sixth-instar armyworms

Activities are means of four determinations and expressed as nmol/h per mg of protein for synthetase, units/mg of protein for cytochrome *c* oxidase and nmol/min per mg of protein for epoxidase. Fractions are described in the Experimental section. ND, Not detected.

Fraction	Volume (ml)	Total protein (mg)	5-Aminolaevulinate synthetase activity		Cytochrome <i>c</i> oxidase activity		Aldrin epoxidase activity	
			Specific	Relative	Specific	Relative	Specific	Relative
Crude	10	18	6.4	0.32	4.9	0.40	2.0	0.40
P ₁	4	3	3.7	0.19	3.3	0.27	0.33	0.07
S ₁	11	13	12.0	0.60	5.5	0.44	2.4	0.48
P ₂	4	5	20.0	1.0	12.4	1.0	2.2	0.44
S ₂	8	5	5.0	0.25	ND		4.0	0.80
P ₃	4	3	—		—		5.0	1.0
S ₃	8	5	—		—		ND

Fig. 6. Variation of 5-aminolaevulinate synthetase activity with age in the last larval instar of the southern armyworm

○, Larvae fed on a control diet; ●, larvae continuously fed on a diet containing 2000 p.p.m. of pentamethylbenzene; □, larvae continuously fed on a diet containing 2000 p.p.m. of diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate.

carboxylate to the larvae (dietary amounts of 2000 p.p.m.) throughout the instar. Both compounds caused a significant induction of synthetase activity, the effect of the latter being greater than that of pentamethylbenzene. The induction occurs with remarkable rapidity in both cases, particularly with diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate, which doubled the midgut synthetase activity within only 4 h. Whereas the activity induced by this compound remains high until the larvae prepare for pupation by clearing their guts at about 130 h, the activity induced by pentamethylbenzene declines after passing through a maximum at about 25 h. Both compounds are also good inducers of microsomal mixed-function oxidase activity in the southern armyworm (Brattsten & Wilkinson, 1973), and, as shown in Fig. 7, diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate caused a fivefold induction in the *N*-demethylation of *p*-chloro-*N*-methylaniline within 24 h. Fig. 7 also shows that when the inducing diet is replaced by a control diet after 24 h the induced *N*-demethylase activity is maintained at a high value when diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate is the inducer. In contrast, the *N*-demethylation activity induced by pentamethylbenzene rapidly decreased to control values on removal of the inducer from the diet (Brattsten & Wilkinson, 1973).

2-Allyl-2-isopropylacetamide, another known porphyrin agent in mammals, caused a doubling of synthetase and *N*-demethylase activities when fed to larvae at dietary concentrations of 1000 and 500 p.p.m. (Table 2), whereas phenobarbital at a concentration of 2500 p.p.m. in the diet increased synthetase activity by only 28% after 24 h of feeding; the latter com-

pound, however, caused a fourfold increase in *N*-demethylase activity under identical conditions.

Induction of synthetase activity by pentamethylbenzene was completely reversed when 10 p.p.m. of puromycin was administered in the same diet. Diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate had no noticeable effect *in vitro* on the synthetase activity, but 1 mM-2-allyl-2-isopropylamide caused approx. 43% inhibition.

Discussion

The optimum conditions for measurement of synthetase activity in armyworm midgut preparations differ considerably from those reported for mammalian-enzyme assays. The major difference in the insect system is its sensitivity to excess of EDTA, pyridoxal phosphate and glycine. Beattie & Stuchell (1970) report a similar inhibition of synthetase activity by excessive concentrations of pyridoxal phosphate in incubations with rat liver mitochondrial preparations. The K_m values for glycine (17 mM) and *S*-succinyl-CoA (20 μ M) with the armyworm enzyme are similar to those (11 mM and 70 μ M respectively) reported for the rat liver enzyme (Scholnick *et al.*, 1972). However, the concentration of *S*-succinyl-CoA required for optimum activity in the armyworm preparation (192 μ M) is much higher than the 0.5–1.0 μ M used by Tschudy *et al.* (1964) in incubations with rat liver. Whereas the armyworm enzyme is active over a fairly narrow range of pH (6.5–7.5), pH values used in incubations with mammalian tissues

Fig. 7. Variation in activity of microsomal *N*-demethylation of *p*-chloro-*N*-methylaniline with age in crude homogenates of midguts from last larval instar of the southern armyworm

○, Larvae fed on a control diet without addition of chemicals; ●, larvae receiving a diet containing 2000 p.p.m. of pentamethylbenzene for the first 24 h after the moult, and subsequently a control diet; □, larvae fed on a diet containing 2000 p.p.m. of diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate for the first 24 h after the moult, and subsequently a control diet.

Table 2. Effect of chemicals on 5-aminolaevalinate synthetase activity and microsomal *N*-demethylation of *p*-chloro-*N*-methyl-aniline administered continuously to sixth-instar larvae of the southern armyworm in an artificial diet

Data are given as percentage of control activities. For the synthetase, control activities are: 12.0 ± 1.42 at 6h; 20 ± 1.76 at 24h; 6.3 ± 0.87 at 72h (nmol/h per mg of protein). The *N*-demethylase control activities are 1.90 ± 0.23 at 6h, 5.54 ± 0.48 at 24h and 7.26 ± 0.89 at 72h (nmol/min per mg of protein).

Chemical (concentration in the diet)	5-Aminolaevalinate synthetase activity			<i>N</i> -Demethylase activity		
	6h	24h	72h	6h	24h	72h
None	100	100	100	100	100	100
Pentamethylbenzene (2000p.p.m.)	183	175	300	247	450	300
Phenobarbital (2500p.p.m.)	142	125	367	233	305	160
Diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-carboxylate (2000p.p.m.)	260	230	700	277	381	—
2-Allyl-2-isopropylacetamide						
2000p.p.m.	167	140	—	—	180	180
1000p.p.m.	—	200	—	—	210	200
500p.p.m.	—	200	—	—	120	200
Pentamethylbenzene (2000p.p.m.) and Puromycin (10p.p.m.)	—	82	—	—	—	75

range from 6.9 (Tschudy *et al.*, 1964) to 8.0 (Hayashi *et al.*, 1972).

The similar subcellular-distribution patterns of synthetase and cytochrome *c*-oxidase activities (Table 1) clearly establish that the former is associated mainly with the mitochondria. However, the substantial epoxidase activity in the mitochondrial (P_2) fraction (Table 1) indicates that this fraction is contaminated with microsomal material which is presumably devoid of synthetase activity.

The practical variables affecting the pyrrole-conversion step were thoroughly studied by using known amounts of authentic 5-aminolaevalinate. The colour intensity produced when the pyrrole was mixed with *p*-dimethylaminobenzaldehyde at 100°C increased significantly with incubation time up to 25min and thereafter decreased. Since the pyrrole concentration is maximum after 25min at 100°C, but is unstable to prolonged heat exposure, this becomes a critical factor in the reproducibility of the synthetase assays. The extinction coefficient obtained after 25min at 100°C ($61 \text{ mm}^{-1} \cdot \text{cm}^{-1}$) was used in the calculations. Other extinction coefficients reported for the pyrrole colour complex are $53 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Granick & Urata, 1963), $56.9 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (DeMatteis, 1971), $64 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Tschudy *et al.*, 1964) and $68 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Mauerszall & Granick, 1956). The absence of colour when a porphobilinogen standard was heated at 100°C for 25min and mixed with Ehrlich's mercury reagent indicates that this pyrrole is also heat-labile and is therefore unlikely to interfere with the colorimetric assay for synthetase activity. In any case there is little or no porphobilinogen produced in the incubation system used. Column chromatography and subsequent t.l.c. separation of the

possible products formed during the incubations also showed that no aminoacetone was produced under the conditions used.

In view of the importance of the synthetase in the formation of the haem component of the respiratory cytochromes it was not entirely unexpected that activity was found in all tissues examined. Activity, however, was by far the highest in the midgut which is the tissue most active in microsomal mixed-function oxidation (Krieger & Wilkinson, 1969).

In normal (uninduced) larvae synthetase activity is highest in the early part of the instar (10–30h of age) (Fig. 6), whereas *N*-demethylase activity does not attain a maximum value until considerably later (Fig. 7). This is almost identical with the patterns of synthetase activity and amounts of cytochrome *P*-450 observed in rats after treatment with phenobarbital (Remmer, 1971) and indicates that synthetase activity precedes the formation of cytochrome *P*-450 and microsomal oxidase activity.

Pentamethylbenzene and diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate are the most effective inducers of armyworm synthetase activity; 2-allyl-2-isopropylacetamide and phenobarbital are less effective (Table 2). Because of the age-dependent changes in enzyme activity in control larvae the actual degree of induction depends on the time at which it is measured (Fig. 6). All four compounds are good inducers of armyworm microsomal oxidases (Table 2). The induction of synthetase activity was blocked by incorporation of 10p.p.m. of puromycin in the diet indicating the involvement of protein synthesis *de novo*. In mice, diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate and 2-allyl-2-isopropylacetamide are reported to be good inducers of syn-

thetase activity, although only the latter causes a substantial increase in microsomal oxidase activity (Wada *et al.*, 1968). Consequently, there appears to be a species difference in response to these two compounds. The highest concentration of 2-allyl-2-isopropylacetamide may well have reached a toxic level to the larvae, causing the observed decreased induction of both synthetase and *N*-demethylase activities (Table 2).

In contrast with the induction of both synthetase and *N*-demethylase activities with diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate, that caused by pentamethylbenzene is relatively short-lived. A possible explanation for this is that pentamethylbenzene is more readily metabolized by microsomal mixed-function oxidation and consequently is available over a shorter period of time.

The compounds diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate, 2-allyl-2-isopropylacetamide and pentamethylbenzene cause induction of both synthetase and *N*-demethylase activities. Phenobarbital, however, causes a large induction of *N*-demethylase activity but has little effect on the synthetase. This is in general agreement with previous reports (De Matteis & Gibbs, 1972) and clearly indicates that induction of synthetase activity is not a prerequisite for enhanced microsomal oxidase activity.

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