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 (20nmol/h per mg of protein) occurs 18–24h 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 pentamethylnitrorene, 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 pentamethylnitrorene. 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 characterize 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**

*Chromicals.* 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. Pentamethylnitrorene 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-di-methanonaphthalene) and its 6,7-epoxide (dieldrin)
were provided by Shell Development Co., Modesto, Calif., U.S.A. [U-14C]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 3 min at 1000g in a chilled centrifuge. The pellet was rehomogenized in 0.9% NaCl and re-centrifuged as above. The combined supernatants (S1) were then centrifuged for 10 min at 13000g (r, 7.5 cm) at 2°C in the IEC model B-20A centrifuge with a fixed-angle head (874) and the pellet (P2) resuspended in 0.9% NaCl was used for measurements of 5-aminolaevulinate synthetase activity.

The microsomal pellets (P3) obtained by centrifugation of a postmitochondrial supernatant for 1h at 100000g (r, 4.5 cm) 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-aminolaevulinate 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-aminolaevulinate synthetase activity are reported in this paper. The incubation mixture finally adopted for optimum synthetase measurements consisted of 180 µmol of sodium phosphate buffer, pH 7.2, 100 µmol of glycine, 0.80 µmol of EDTA, 0.50 µmol of pyridoxal phosphate, 0.58 µmol of succinyl-CoA and 0.4–0.7 mg of protein in a total volume of 3.0 ml. The reactions were left to proceed aerobically for 30 min at 40°C in a Lab-Line Instruments metabolic incubator and were terminated by the addition of 2.0 ml of cold (15°C) 10% (w/v) trichloroacetic acid. After deproteinization, 0.1 ml of 2.4-pentanediol was added to 3.0 ml of the acid supernatant and the pH was adjusted to 5.6 with 0.9 ml of 1 M-sodium acetate buffer. This mixture was held at 100°C in a Scientific Products Tempblock Module heater for 25 min, and after cooling, 3.0 ml was mixed with an equal volume of modified Ehrlich’s mercury reagent (Urata & Granick, 1963). After 15 min at room temperature (21°C) the extinction of the samples was read at 552 nm 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 1.875 nm, was included with each experiment. Porphobilinogen was measured by mixing 3.0 ml 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 552 nm. In the incubation with [14C]glycine, 60 µmol of unlabelled glycine and 0.5 µCi of [14C]glycine were used instead of the 100 µmol 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 (2 cm x 1 cm) of Dowex 1 anion exchange resin (X8; 200–400 mesh) converted into the acetate form with sodium acetate (0.5–2 M). The eluate was evaporated under a stream of dry N2, redissolved in methanol and applied to a t.l.c. plate ( precoated, 0.25 mm thick, silica gel, 60F-254; EM Reagents, Darmstadt, Germany). The plate was developed in a solvent system of methyl acetate–propan–2-ol–7.1 m-NH3 (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 YSI 4004 Clark oxygen probe connected to a Gilson Oxymeter XM at 30°C in an incubation system containing 140 µmol of sodium phosphate buffer, pH 7.2, 81 µmol of cytochrome c, 14 µmol of l-ascorbic acid and 0.1 mg of protein in a total volume of 1.5 ml. The results are expressed as percentage of O2 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 (±1.5h) 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-aminolaevulinate synthetase activity in vitro in the mitochondrial pellet (P2) 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 optimum buffer concentration in the incubation mixture for enzyme activity was at about 180 μmol/3.0 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 μmol/incubation and decreased sharply in incubation mixtures containing less than 0.5 μmol or more than 1.25 μmol. The pyridoxal phosphate concentration was also critical, and gave maximum activity at 0.5 μmol/incubation (Fig. 1d). The addition of more than 100 μmol 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-aminolaevulinate 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.

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![Graphs](image)

**Fig. 1. Incubation conditions for 5-aminolaevulinate synthetase**

(a) Effect of pH. ○, Tris–HCl buffer; ●, 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.
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 M⁻¹ 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.I.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 Rf values of [¹⁴C]glycine and porphobilinogen were 0–0.10. The 5-aminolaevulinate–pyrrole migrated with the Rf value of 0.48–0.50, whereas the aminoacetone–pyrrole closely followed the solvent front (Rf value of 0.90–1.0). T.I.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 [¹⁴C]glycine.

The data were reproducible and included the linear range of 0.25–0.75 mg of protein/incubation (Fig. 2). The apparent molar extinction coefficient (εapp) for the colour complex formed by the 5-aminolaevulinate–pyrrole and the modified Ehrlich’s mercury reagent were confirmed by the results from incubations with [¹⁴C]glycine.

Two final concentrations of 5-aminolaevulinate (25 and 400 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 (1.10 nmol/h per mg of protein) only after the addition of 200 nmol 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 followed 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 10 min at 13000g\(\text{av}\). 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. 5 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 15 h of the sixth instar by which time it had attained a maximum specific activity of about 20 nmol/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.

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

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**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.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>5-Aminolaevulinate synthetase activity</th>
<th>Cytochrome c oxidase activity</th>
<th>Aldrin epoxidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specific</td>
<td>Relative</td>
<td>Specific</td>
</tr>
<tr>
<td>Crude</td>
<td>10</td>
<td>18</td>
<td>6.4</td>
<td>0.32</td>
<td>4.9</td>
</tr>
<tr>
<td>P1</td>
<td>4</td>
<td>3</td>
<td>3.7</td>
<td>0.19</td>
<td>3.3</td>
</tr>
<tr>
<td>S1</td>
<td>11</td>
<td>13</td>
<td>12.0</td>
<td>0.60</td>
<td>5.5</td>
</tr>
<tr>
<td>P2</td>
<td>4</td>
<td>5</td>
<td>20.0</td>
<td>1.0</td>
<td>12.4</td>
</tr>
<tr>
<td>S2</td>
<td>8</td>
<td>5</td>
<td>5.0</td>
<td>0.25</td>
<td>ND</td>
</tr>
<tr>
<td>P3</td>
<td>4</td>
<td>3</td>
<td>—</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>S3</td>
<td>8</td>
<td>5</td>
<td>—</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

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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 4h. Whereas the activity induced by this compound remains high until the larvae prepare for pupation by clearing their guts at about 130h, the activity induced by pentamethylbenzene declines after passing through a maximum at about 25h. 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 24h. Fig. 7 also shows that when the inducing diet is replaced by a control diet after 24h 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 porphyric 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 24h of feeding; the latter compound, 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 noticable effect in vitro on the synthetase activity, but 1mm-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 Ke values for glycine (17 millimolar) and S-succinyl-CoA (20 micromolar) with the armyworm enzyme are similar to those (11 millimolar and 70 millimicromolar) 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 millimolar) is much higher than the 0.5-1.0 micromolar 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

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

O, 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.

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**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

O, Larvae fed on a control diet without addition of chemicals; ●, larvae receiving a diet containing 2000 p.p.m. of pentamethylbenzene for the first 24h 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 24h after the moult, and subsequently a control diet.
Table 2. Effect of chemicals on 5-aminolaevulinate 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).

<table>
<thead>
<tr>
<th>Chemical (concentration in the diet)</th>
<th>5-Aminolaevulinate synthetase activity</th>
<th>N-Demethylation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6h</td>
<td>24h</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pentamethylbenzene (2000 p.p.m.)</td>
<td>183</td>
<td>175</td>
</tr>
<tr>
<td>Phenobarbital (2500 p.p.m.)</td>
<td>142</td>
<td>125</td>
</tr>
<tr>
<td>Diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-carboxylate (2000 p.p.m.)</td>
<td>260</td>
<td>230</td>
</tr>
<tr>
<td>2-Allyl-2-isopropylacetamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000 p.p.m.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 p.p.m.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 p.p.m.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentamethylbenzene (2000 p.p.m.) and Puromycin (10 p.p.m.)</td>
<td>—</td>
<td>82</td>
</tr>
</tbody>
</table>

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 epoxide activity in the mitochondrial (P2) 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-aminolaevulinate. The color intensity produced when the pyrrole was mixed with p-dimethylaminobenzaldehyde at 100°C increased significantly with incubation time up to 25 min and thereafter decreased. Since the pyrrole concentration is maximum after 25 min 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 25 min at 100°C (61 mM⁻¹ cm⁻¹) was used in the calculations. Other extinction coefficients reported for the pyrrole colour complex are 53mM⁻¹ cm⁻¹ (Granick & Urata, 1963), 56.9mM⁻¹ cm⁻¹ (De Matteis, 1971), 64mM⁻¹ cm⁻¹ (Tschudy et al., 1964) and 68mM⁻¹ cm⁻¹ (Mauerzall & Granick, 1956). The absence of colour when a porphobilinogen standard was heated at 100°C for 25 min 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 aminocetone 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.

References


1975