MICROSOMAL HYDROXYLATION OF ANILINE IN THE SOUTHERN ARMYWORM, SPODOPT ERA ER/ DAN IA

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MICROSOMAL HYDROXYLATION OF ANILINE IN THE SOUTHERN ARMYWORM, *SPODOPTERA ERIDANIA*

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Abstract-Microsomal hydroxylation of aniline in midguts of the southern armyworm, *Spodoptera eridania*, was found to differ in its requirements for optimal *in vitro* activity from those of armyworm and rat liver pchloro N-methylaniline N-demethylase and rat liver aniline hydroxylase. Apparent K_m values for armyworm aniline hydroxylase were substantially higher than those for rat liver aniline hydroxylase and the N-demethylase enzymes. The armyworm aniline hydroxylase was inhibited by several mixed-function oxidase inhibitors as well as by aldrin and nicotine and was highly inducible by pentamethylbenzene and phenobarbital.

INTRODUCTION

As A RESULT of intensive studies in recent years it has been established that the midgut of the southern armyworm *(Spodoptera eridania)* contains an active microsomal mixed-function oxidase (MFO) system capable of catalyzing a variety of reactions including the epoxidation of aldrin (KRIEGER and WILKINSON, 1969), the alicyclic hydroxylation of dihydroisodrin (KRIEGER and WILKINSON, 1971), and the N-demethylation of pchloro N-methylaniline (KRIEGER and WILKINSON, 1970). The system contains high titres of cytochrome P-450 and NADPH-cytochrome c reductase, is highly inducible (BRATTSTEN and WILKINSON, 1973) and in all important aspects reported to date closely resembles the mammalian liver MFO system.

During an earlier investigation in this laboratory (WILKINSON and HICKS, 1969) it was observed that the ability of armyworm midgut microsomes to catalyze the p-hydroxylation of aniline was considerably less than would be expected from its high oxidase activity towards other substrates. In view of this observation and the fact that aniline hydroxylation has to date received little attention in insects it was considered of interest to carry out an in-depth comparison of aniline hydroxylation with N-demethylation in the armyworm and with these activities in rat liver microsomes.

MATERIALS

Animals

A colony of the southern armyworm was maintained as described by KRIEGER and WILKINSON (1969) facilitated by a periodical supply of eggs from Niagara Chemical Division, FMC Corp., Middleport, N.Y. Last (sixth) instar larvae were used throughout this investigation. Male rats (100 to 150 g) of Sprague-Dawley descent were purchased from Blue Spruce Farms, Altamont, N.Y.

Chemicals

Aldrin, 1,2,3,4,10,1O-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo,exo-5,8-dimethanonaphthalene was donated by Shell Development Co., Modesto, CA. Glucose-6-phosphate (G-6-P), G-6-P-dehydrogenase, p-chloro N-methylaniline, p-chloroaniline and NADP, disodium salt, were purchased from Calbiochem, La Jolla, CA, Pentamethylbenzene, p-dimethylaminobenzaldehyde, aniline HCl, *p*-aminophenol HCl, and nicotine were from Eastman Kodak Co., Rochester, N.Y. and 3-methylcholanthrene was from Sigma Chemical Co., St. Louis, MO. Phenobarbital was from Mallinckrodt Chemical Works, St. Louis, MO. and piperonyl butoxide from Chem Service, Westchester, P.A. A sample of 5,6-dichlorobenzothiadiazole was a gift from Dr. Hugh Davies of Shell Research Limited, Woodstock Agricultural Research Centre, Sittingbourne, Kent, U.K. The 1-nonylimidazole was synthesized as described previously (WILKINSON *et al.*, 1974). Alphacel, cholesterol (USP), linolenic acid (55%), salt mixture W, Vanderzant's vitamin mixture, and vitamin free casein were purchased from Nutritional Biochemical Corp., Cleveland, OH., Bacto-agar was from Difeo Laboratories, Detroit, MI. and wheat germ from Kretschmer Wheat Germ Products, Minneapolis, MN.

METHODS

Enzyme preparations

Midguts from groups of several hundred armyworms, fed a semi-defined artificial diet (BRATTSTEN and WILKINSON, 1973) containing 0.2% pentamethylbenzene for 3 days, were used to establish the optimum incubation parameters for aniline hydroxylase activity. The guts were dissected out, the fore- and hindguts excised and the midguts were sectioned longitudinally to remove the gut contents prior to washing and storage in ice-cold 1.15% KC!. The cleaned midguts were homogenized in a ground glass tube with a motor driven tefion pestle for 60 sec in ice-cold 1.15% KC! and the resulting homogenate was centrifuged at 12,000 *g* for 10min (2°C) in an IEC B-20A centrifuge. The supernatant was filtered through glass wool, the pellet

rehomogenized in 80% of the original volume of fresh 1.15% KC!, and recentrifuged and filtered as before. The combined supernatants were centrifuged for 1 hr at 100,000 *g* in an IEC B-60 preparative ultracentrifuge (2 to 4°C) and the resulting microsomal pellet was suspended in 1.15% KC! at a concentration of 6 to 8 mg protein/ml.

Livers from rats, killed by decapitation, were minced in ice-cold 1.15% KC!, homogenized in a smooth glass tube with a motor driven teflon pestle for 60 sec and microsomal pellets were obtained by fractional centrifugation as described above.

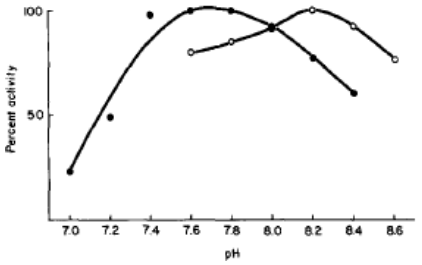

Fig. 1. Aniline hydroxylase activity in midgut microsomes from the southern armyworm (0) and in rat liver micro- somes (e) as a function of the pH of the 0.05 M Tris-HCI buffer. Incubations carried out under otherwise optimal conditions.

Enzyme assays

Microsomal N-demethylation of p-chloro N-methylaniline was assayed as described previously (BRATISTEN and WILKINSON, 1973). Aniline hydroxylase activity in the armyworm midgut microsomes (AMM) was measured in a 5.0 ml incubation mixture consisting of 50 mM Tris-HCI buffer, pH 8.2, 0.1 M KC!, 0.25 mM NADP, 2.4 mM G-6-P, 1.6 units of G-6-P dehydrogenase, 2.0 mM aniline HCI and 3-4 mg microsomal protein. The reactions were initiated by addition of the enzyme and proceeded for 12 min at 32°C with shaking; they were terminated by addition of 2.0 ml 20% TCA. After centrifugation at 12,000 *g* for 15 min a 2.0 ml aliquot of the acid supernatant was mixed with 2.0 ml of a solution of 1.0 g phenol and 2.0 g NaOH in lOO ml water and 2.0 ml of 1M Na_2C0_3. The blue colour developed in 20 min at room temperature and the absorbance at 610 nm was measured against zero-time blanks (SCHENKMAN *et al.*, 1967) in a Unicam SP.800A spectrophotometer with a scale expansion accessory. Product formation was estimated by reference to a standard curve prepared by addition of known amounts of p-aminophenol to incubation mixtures containing denatured (by TCA precipitation) enzyme; the molar extinction coefficient (E_{610}) of the colour complex was 27,125 $M^{-1} cm^{-1}$.

Aniline hydroxylase activity in rat liver microsomes (RLM) was measured in a 5.0 ml incubation system consisting of 50 mM Tris-HCI buffer pH 7.6, 2.4 mM G-6-P, 1.6 units of G-6-P dehydrogenase, 15.4 mM KC!, 0.75 mM NADP, 5.0 mM $MgCl_2$, 0.73 mM aniline-RC! and 2-3 mg microsomal protein. The incubations proceeded for 20 min at 32°C with shaking. The subsequent assay procedure was as described above.

All incubations were carried out in duplicate and the experiments were repeated two or three times. Enzyme activities are expressed as nmoles of product formed/min/mg protein.

Concentration of cytochrome P-450 was measured in 0.6 M phosphate buffer, pH 7.6, and glycerol (1/1, v/v) as the carbon monoxide complex after reduction with dithionite in an Aminco-Chance DW-2 spectrophotometer (OMURA and SATO, 1964).

Protein concentrations were measured by the Biuret method (CLARK, 1964) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Incubation conditions

The optimum incubation conditions for aniline hydroxylase described in the Methods section were arrived at as a result of detailed studies of the effect of pH, protein concentration, time of incubation and concentrations of the components of the reaction mixture.

With Tris-HCl buffer (50 mM) aniline hydroxylase activity was highest over a rather broad range around pH 8.2 in AMM and 7.6 in RLM (Fig. 1). The use of phosphate buffer (50 mM) gave identical curves at a slightly reduced activity level with both enzymes. The two other well characterized MFO reactions used routinely in this laboratory, N-demethylation of p-chloro N-methylaniline and epoxidation of aldrin have the lower pH optimum of 7.8 with AMM (KRIEGER and WILKINSON, 1969, 1970). These two reactions in RLM have maximum activity at pH 7.4. CHANG and HODGSON (1975) report a pH of 7.9 to be suitable for several housefly MFO reactions including the hydroxylation of aniline.

At 30 to 32°C activity increased linearly with protein concentration up to 3 to 4 mg per incubation with both enzymes and within this range the reaction rate was constant for 12 min with AMM and for 20 min with RLM.

As shown in Table 1 the presence of $MgC1_2$ caused a stimulation of hydroxylase activity in RLM but had an adverse effect on activity in AMM. A combination

Table 1. Effect of components on the reaction mixture on *in vitro* aniline hydroxylase activity in armyworm midgut and rat liver microsomes. Other components were at optimum concentrations

Component	Concentration (mM)	Percent activity AMM	RLM
$MgCl_2$ +	0	100	77
27.6 mM KC!	5	74	95
	10	69	100
	20	60	91
KC!	15.4	63	
	27.6	80	
	100	100	
	200	94	
KC!+ $MgCl_2$	15.4 + 5		100
	27.6 + 5		93
	15.4 + 17.3		89
	32.6 + 0		67

Table 2. Distribution of aniline hydroxylase activity in centrifugal fractions of midgut from sixth instar armyworms fed a diet containing 0.2% pentamethylbenzene for 3 days

Fraction*	Total protein (mg)	Specific activity (nmole/mg protein/min)	Relative activity
Crude homogenate	63		0.83
P12,ooo	303		0.46
S12,ooo	313		1.10
P100.ooo	70		4.12
S100,ooo	187		0.08

* P12.ooo = 12,000 *g* pellet; S12.000 = 12,000 *g* supernatant; etc.

of a low concentration (15.4 mM) of KCl and 5 mM of $MgCl_2$ gave best activity with RLM whereas AMM needed a high concentration (100 mM) of KCl for maximum activity and absence of magnesium ions. This amount of KCl represents a 3.6 times higher ionic strength in the incubation medium than is required for measurements of epoxidation and N-demethylation in AMM.

There was also a difference in the concentration of NADP needed for maximum hydroxylase activity, AMM requiring 0.25 mM and RLM the higher level of 0.75 mM. With AMM N-demethylase and aldrin epoxidase maximum activity is attained at a concentration of NADP of only 0.05 mM.

In view of the possibility that the product, p-aminophenol, might be unstable (CHASTRIL and WILSON, 1975) or susceptible to enzymic conjugation in the incubation system, experiments were conducted in which p-aminophenol (100 µmole) was incubated for 30 min at 32°C in reaction mixtures containing 12.9 mg crude armyworm midgut protein. Under these conditions 94% of the product measured in appropriate blanks (without any protein) was recovered.

Subcellular localization

A study of the subcellular distribution of aniline hydroxylase in armyworrn midgut homogenates (Table 2) showed the specific activity (4.12 nmole/min/mg protein) of the microsomal fraction (P100,ooo) to be almost 4-fold higher than that in any other fraction. Of the total activity in the crude homogenate (525 nmole/min), almost 27% (139 nmole/min) remained in the twice homogenized and centrifuged heavy pellet ($P_{12.000}$) containing cell fragments, nuclei and mitochondria. Of the activity in the combined 12,000 *g* supernatants ($8_{12.000}$) almost 89% (288 nmole/min) was recovered in the microsomal fraction ($P_{100.000}$); less than 5% remained unsedimented. This pattern of distribution of the activity is virtually identical to those previously reported for AMM N-demethylase and aldrin epoxidase (BRATTSTEN and WILKINSON, 1973).

Age-activit y relationship and inducibility of armyworm M FO's

Table 3 shows the age-dependent variation in hydroxylase and N-demethylase activities in midgut microsomes from control and induced sixth instar armyworrns. In control armyworms the rate of increase in activity with larval age *is* quite similar with both enzymes although the actual level of aniline hydroxylase activity remains 5 to 7-fold lower than that of N-demethylase throughout. The age related rate of increase in activity of both enzymes persists in the presence of the inducing agents pentamethylbenzene and phenobarbital, although aniline hydroxylation is induced approximately 2-fold more than N-demethylation. In spite of this, aniline hydroxylase activity never attains the high levels observed with *N*-demethylation. Pentamethylbenzene is a more effective inducer of aniline hydroxylase activity than of N-demethylase and caused a 6- to 7-fold induction of the former compared to a less than 4-fold increase in the latter. Phenobarbital, a generally less effective inducer of AMM enzymes increased both activities to a similar degree. Under identical treatment condi-

Table 3. Variation of aniline hydroxylase and N-demethylase with age in midgut microsomes of sixth instar armyworms fed a control diet or diets containing 0.20% pentamethylbenzene or 0.25% phenobarbital

Pretreatment	Age of larvae 24 hr	48 hr	72 hr
		Aniline hydroxylase	
Control	0.32 ± 0.04* (lOO)t	0.41 ± 0.03 (100)	0.74 ± 0.04 (100)
Pentamethylbenzene	1.66 ± 0.12 (519)	2.96 ± 0.21 (722)	4.45 ± 0.50 (601)
Phenobarbital	1.16 ± 0.12 (363)	1.34 ± 0.14 (327)	2.52 ± 0.15 (341)
		N-demethylase	
Control	2.15 ± 0.20 (100)	2.40 ± 0.25 (100)	3.85 ± 0.40 (100)
Pentamethylbenzene	6.87 ± 0.75 (320)	8.17 ± 1.00 (340)	14.56 ± 1.20 (378)
Phenobarbital	5.35 ± 0.30 (249)	6.10 ± 0.70 (254)	12.50 ± 1.50 (325)

* Specific activities (nmole/min/mg protein) ± standard error of the mean; $n = 3$ or 4.

t Numbers in parentheses are % of control activity showing induction.

Table 4. Kinetic parameters of aniline hydroxylase and p-chloroN-methylaniline N-demethylase in control and induced armyworm midgut and rat liver microsomes

Animal	Inducer*	Aniline hydroxylase		N-demethylase	
		Km (μM)	Vmax (nmoles/min/ rrig protein)	*Km* (μM)	vmax (nmoles/min/ mg protein)
Rat	none	44 ± 1	0.36 ± 0.01	47 ± 4	1.33 ± 0.04
Rat	Pentamethylbenzene	36 ± 1	0.88 ± 0.12	18 ± 1	0.89 ± 0.04
Rat	Phenobarbital	104 ± 18	0.87 ± 0.06	31 ± 1	10.74 ± 0.07
Rat	3-methylcholanthrene	108 ± 8	0.45 ± 0.01	64 ± 5	2.01 ± 0.07
Armyworm	none	724 ± 31	0.58 ± 0.04	265 ± 16	3.70 ± 0.16
Armyworm	Pentamethylbenzene	908 ± 93	4.24 ± 0.32	270 ± 7	13.58 ± 0.28
Armyworm	Phenobarbital	675 ± 43	1.72 ± 0.14	237 ± 29,	12.38 ± 0.85
Armyworm	3-methylcholanthrene	723 ± 166	0.72 ± 0.10	381 ± 33	5.16 ± 0.22

* Pentamethylbenzene administered to rats by i.p. injection of 50 mg/kg in corn oil 24 and 36 hr before killing; to armyworms for 3 days in diet at 0.20%. Phenobarbital administered to rats for 10 days at 0.1% in drinking water; to armyworms for 3 days in diet at 0.25%. 3-methycholanthrene administered to rats by i.p. injection of 20 mg/kg in corn oil 24 and 36 hr before killing and to armyworms for 3 days in diet at 0.03%.

tions, pentamethylbenzene causes a 208% increase in the level of the cytochrome P-450 compared to an increase of 70% after phenobarbital treatment.

Kinetic characteristics

Considering the complex, membrane-bound nature of the enzyme and the impurity of the microsomal fraction, kinetic parameters of MFO catalyzed reactions can at best be regarded only as gross indicators of reaction characteristics. Despite these limitations apparent kinetic characteristics of aniline hydroxylase and N-demethylase in AMM and RLM prepared from control animals and from animals pretreated with several MFO-inducing chemicals are shown in Table 4.

With the exception of phenobarbital induced RLM, where pronounced inhibition was observed at high substrate concentration, the Lineweaver-Burk double reciprocal plots were linear with correlation coefficients higher than 0.945. The range of substrate concentrations used was 0.17-2.0 mM for aniline and 0.02--0.38 for p-chloro N-methylaniline. In control animals RLM appears to have a considerably greater affinity (lower *Km* values) than AMM for both reactions although Vmax values for AMM are either equal to or greater than those for RLM. None of the inducers (pentamethylbenzene, phenobarbital, 3-methylcholanthrene) appeared to cause any substantial change in the *Km* values for either substrate, although the results with RLM were more variable than those with AMM. There were, however, large differences in Vmax values. The data show that 3-methylcholanthrene had little effect on the vmax of either enzyme in AMM and RLM. Pentamethylbenzene had a remarkable inductive effect on the AMM enzymes increasing the aniline hydroxylase Vmax 7-fold. In contrast, pentamethylbenzene caused a slight decrease in N-demethylase activity in RLM whilst increasing the aniline hydroxylase Vmax more than 2-fold. Phenobarbital was particularly effective with the RLM N-demethylase where it caused an almost 10-fold increase in vmax.

The variation in *Km* values in RLM from induced animals may support the proposition of the existence of several different MFO enzymes catalyzing the hydroxylation of the same substrate molecule by different mechanisms in the rat (RICKERT and Foms, 1970; ANDERS, 1972) although the data obtained with AMM do not support a similar conclusion for the armyworm MFO enzymes. The most obvious difference between the rat and the armyworm is in their response to pentamethylbenzene. Possibly this compound has toxic effects on the rats at the concentration used.

Inhibition

I_{50} values were obtained from plqts of percent inhibition *versus* log concentration of the inhibitor, using the incubation mixtures described above which gave the highest initial velocities, to which five to seven different concentrations of the inhibitor was added. For aniline hydroxylase activity in pentamethylbenzene induced AMM the 1_{50} values were 7.2 x 10^{-6} M for piperonyl butoxide ($r = 0.988$), 5.1 x 10^{-7} M for 1-nonylimidazole ($r = 0.996$) and 1.0 x 10^{-5} M for 5,6-dichloro-1,2,3-benzothiadiazole ($r = 0.999$). The sensitivity of the AMM hydroxylase to these established MFO inhibitors is very similar to that of pentamethylbenzene induced as well as control AMM aldrin epoxidase (WILKINSON *et al.*, 1974; GIL and WILKINSON, 1976).

The addition of 100 μM nicotine to aniline hydroxylase incubations of AMM resulted in 2 to 4 fold inhibition of hydroxylase activity as indicated by the reduction in Vmax, Table 5. Nicotine is a more effective inhibitor of aniline hydroxylase than aldrin. With pentamethylbenzene induced aniline hydroxylase there is no change in K_m that can be attributed to

Table 5. *In vitro* i;ffect of aldrin and nicotine on aniline hydroxylase activity in midgut microsomes from armyworms fed a 0.20% pentamethylbenzene or a 0.25% phenobarbital diet for 3 days

Inducer	Inhibitor (μM)	K_m (μM)	V_{max} (nmole/min/mg protein)
Pentamethylbenzene	None	908 ± 93	4.24 ± 0.32
Pentamethylbenzene	Nicotine (10)	939 ± 195	1.16 ± 0.14
Pentamethylbenzene	Aldrin (100)	1112 ± 109	1.85 ± 0.13
Phenobarbital	None	675 ± 43	1.72 ± 0.14
Phenobarbital	Nicotine (10)	397 ± 79	0.69 ± 0.05
Phenobarbital	Aldrin (100)	466 ± 76	0.39 ± O.Q3

the inhibitors. Both nicotine and aldrin effected clear-cut non-competitive inhibition of aniline hydroxylase. This might be expected in the case of aldrin which gives a type I binding spectrum with cytochrome P-450 (KULKARNI *et al.*, 1975) and presumably combines by a different mechanism (CHAPLIN and MANNERING, 1970) with the cytochrome than compounds showing type II spectra such as nicotine and aniline (SCHENKMAN *et al.*, 1967; KULKARNI *et al.*, 1975). The non-competitive inhibition by nicotine might indicate the existence also in AMM of more than one enzyme 'catalyzing the hydroxylation of aniline although no proof positive has yet been obtained for this possibility. The double reciprocal Lineweaver-Burk type plots showed in all cases straight lines although NAKANISHI *et al.* (1972) reported that addition of type I binders to rat and mouse liver aniline hydroxylase assays change the appearance of the straight lines to curves and result in rather complicated types of inhibition. Hydroxylation of naphthalene in microsomes obtained from house flies was also reported to show a mixed type of inhibition by piperonyl butoxide (HANSEN and HODGSON, 1971).

Effect of acetone

Acetone has been reported to have a remarkable *in vitro* stimulatory effect on aniline hydroxylation in rat liver microsomes (ANDERS, 1968; VAINIO and HANNINEN, 1972) and to be less stimulatory with dog or mouse liver microsomal aniline hydroxylation. The aniline hydroxylase activity in AMM is totally insensitive to the presence of acetone in the incubation mixtures: 0.05 M acetone resulted in 104% of control activity and 0.05 M in i06% of control activity.

The data reported show that although AMM aniline hydroxylase has most of the characteristics associated with a typical MFO enzyme it differs in several requirements for optimum *in vitro* activity from other armyworm MFO reactions. In control armyworms, as well as in rats, aniline hydroxylase activity is considerably lower than that of N-demethylase or aldrin epoxidase, a condition which is also reflected in the higher *Km* value of AMM aniline hydroxylase. In armyworms, but not in rats, aniline hydroxylase is much more inducible than N-demetbylase. The results further support the theories concerning the multiple nature of MFO enzymes and their responsiveness to external factors. It is not obvious what the possible biological significance of the lower hydroxylation capacity may be. Alicyclic hydroxylation capacity has also been reported to be comparatively low in some cases (KRIEGER and WILKINSON, 1971; GILBERT and WILKINSON, 1974).

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