MONOOXYGENASE DRUG METABOLIZING ACTIVITY IN $CaCl_2$-AGGREGATED HEPATIC MICROSOMES FROM RAT LIVER

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Summary

Rapidly sedimenting $CaCl_2$-aggregated microsomes from rat liver were found to catalyze the demethylation of aminopyrine (AP) and p-chloro-N-methylaniline (PCMA) and the hydroxylation of Δ^1-tetrahydrocannabinol (Δ^1-THC). The demethylation of AP required NADPH and O_2 and was inhibited by carbon monoxide. This enzymatic activity was stimulated by the prior treatment of immature male rats with phenobarbital. The specific enzymatic activity of the $CaCl_2$-aggregated microsomes was similar to that obtained with normal (100,000 g centrifuged) microsomes. It is suggested that the $CaCl_2$-aggregation yields functionally-intact microsomes.

The mammalian liver is known to possess a monooxygenase enzyme system which in the presence of oxygen and NADPH catalyzes the hydroxylation of a variety of substances, such as drugs and certain endogenous steroids. Furthermore the hepatic monooxygenase activity can be enhanced by the administration to animals of a variety of structurally unrelated compounds, among these barbiturates and chlorinated hydrocarbon insecticides (1, 2). This enzymatic property of liver endoplasmic reticulum is found to be associated with the microsomal fraction usually obtained as a pellet after a 105,000 g centrifugation of liver preparations.

Recently Kamath *et al* (3) described a novel procedure for the preparation of liver microsomes. This procedure involves the use of $CaCl_2$ in the homogenization medium which produces an aggregation of the microsomes permitting their isolation by a low speed centrifugation (1,500 g for 10 min). On the other hand preparation

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of the "normal" microsomes in the same homogenization medium but in the absence of $CaCl_2$ required centrifugation at about 105,000 g for 1 hour.

The present study examines the activity of the monooxygenase system from rat livers in the $CaCl_2$-aggregated microsomes (1500 g-pellet) and compares this activity with that of "normal" microsomes. The monoxygenase activities examined were the demethylation of aminopyrine (AP), demethylation of p-chloro-N-methyl-aniline (PCMA) and the oxidative metabolism of Δ^1-tetrahydrocannabinol (Δ^1-THC). These compounds were selected to represent type I and II substrates*. Furthermore the effect of induction of the monooxygenase by phenobarbital on the magnitude of AP demethylation by this enzyme in the $CaCl_2$-aggregated and "normal" microsomes was investigated.

Materials and Methods

Animals: Sprague-Dawley male rats (60-80 g) were obtained from Charles River Breeding Laboratories, N. Wilmington, Mass. They were fed Purina Chow and water *ad lib.*

Compounds: Aminopyrine was obtained from Aldrich Chemical Co.; p-Chloro-N-methyl-aniline and p-chloroaniline were obtained from Calbiochem; Glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase (Type V from baker's yeast) were purchased from Sigma Chemical Co.; trans-Δ^1-tetrahydrocannabinol (Δ^1-THC) and ^{14}C-Δ^1-THC (labeled at positions 4' and 6') were obtained from the National Institute of Mental Health. Phenobarbital (Na) is a product of Mallinckrodt Chemical Works and SKF 525A (β-diethylaminoethyl diphenylpropylacetate) is a product of Smith Kline and French Co. All other compounds used were of reagent grade quality.

Preparation of Microsomes: Rats were decapitated, livers immediately removed and placed on ice. Livers were homogenized in the appropriate solutions (see below) using 5 ml per gram of wet weight liver, with a Potter-Elvehjem glass homogenizer

*The addition of certain compounds to microsomes elicits spectral changes which are thought to reflect the binding to the enzyme (4, 5). Two major types of spectral changes have been obtained: type I (minimum at about 420 nm and maximum at about 390 nm) is produced with Δ^1-THC (6, 7a,b) and AP (4), whereas aromatic amines such as PCMA (8), yield type II (min. at about 390 and max. at 420-430 nm).

equipped with a Teflon plunger. In some of the studies livers from the same animals were utilized for the two types of microsomal preparations.

$CaCl_2$-aggregated microsomes: The solutions used were as previously described (3). All operations were carried out at 0-4°C. The homogenizing medium was composed of sucrose (0.25 M), Tris-Cl, pH 7.5 (0.05 M), $MgCl_2$ (0.005 M), KCl (0.025 M), and $CaCl_2$ (0.008 M). The homogenate was centrifuged at 10,000 g for 15 min in a Servall centrifuge. The pellet was discarded and the supernate was diluted 1:6 with a solution containing sucrose (0.0125 M), $MgCl_2$ (0.005 M) and $CaCl_2$ (0.008 M). Centrifugation was carried out at 1,500 g for 10 min. The resultant supernate was discarded and the pellet dispersed in sucrose (0.0125 M) and centrifuged at 1500 g for 10 min. The resulting pellet was next suspended in cold 1.15% aqueous KCl to yield a homogeneous microsomal suspension (5 ml per gram of original liver).

"Normal" microsomes: Livers were homogenized in 0.25 M sucrose. The resulting homogenate was centrifuged at 10,000 g for 15 min and the resulting supernate was centrifuged for 1 hour at 100,000 g in a Spinco model L ultracentrifuge. The resulting pellet was resuspended in fresh 0.25 M sucrose (5 ml/g of liver) and centrifuged at 100,000 g for 1 hour. The final pellet ("normal" microsomes) was suspended in 1.15% KCl (5 ml/g of liver).

Incubations, constituents and conditions: All incubations were carried out in 25 ml Erlenmyer flasks with shaking in a Dubnoff incubator in an atmosphere of air at 37°C (unless otherwise indicated).

1. Aminopyrine demethylation: Tris buffer pH 7.5 (0.05 M), $MgCl_2$ (5 mM), nicotinamide (5 mM), $NADP^+$ (0.5 mM), glucose-6-phosphate (4.5 mM), glucose-6-phosphate dehydrogenase (5 IU), aminopyrine (5 mM) and 0.5-1.0 ml of microsomal suspension in a final volume of 4 ml. Reactions were started by the addition of glucose-6-phosphate dehydrogenase, except when otherwise indicated. After incubating for 20 or 30 minutes, the reaction was terminated by the addition of 1.3 ml of 20% $ZnSO_4$ followed rapidly by 1.3 ml of saturated $Ba(OH)_2$. After brief centrifugation, 1.6 ml of the "Nash" reagent was added to 4 ml aliquots of the supernate and the resulting solutions were heated

for 30 min at 60°C (9); the absorbance was determined at 415 nm. The amount of formaldehyde formed was determined from a standard curve.

2. p-Chloro-N-methylaniline (PCMA) demethylation: Sodium phosphate buffer (0.05 M, pH 7.5), $MgCl_2$ (10 mM), nicotinamide (10 mM), glucose 6-phosphate 9 mM), $NADP^+$ (1 mM), glucose-6-phosphate dehydrogenase (3 IU), PCMA (1.5 mM); reaction was started by the addition of 0.5-1.0 ml of microsomal suspension, the final volume being 2.0 ml. Incubations were carried out for 15 min and the amount of p-chloroaniline formed was determined as previously described (10).

3. Δ^1-THC Oxidation: Phosphate buffer (0.04 M, pH 7.4), $MgCl_2$ (15 mM), NADP (0.4 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (5 units) and 1 ml of the microsomal suspension in a final volume of 5 ml. After brief equilibration at 37°C, the reaction was initiated by the addition of 0.15 ml of a solution of ^{14}C-Δ^1-THC (0.48 µmoles, 47,300 DPM) in ethanol-propylene glycol (1:1). After 30 minutes of incubation, 10 ml of ethyl acetate is added to the reaction mixture with immediate shaking. The extraction of the unreacted Δ^1-THC and the metabolites is carried out by the subsequent vigorous shaking of the mixture for 1 hour. The analysis of the products in the ethyl acetate phase was carried out by thin layer chromatography and autoradiography as previously described (11).

Protein determinations were carried out by the Lowry procedure (12). Cytochrome P-450 was measured as follows: $CaCl_2$-aggregated microsomes (4.8 mg protein) were suspended in 6 ml of 50 mM KCl - 33 mM phosphate buffer, pH 7.7, a few mg of sodium dithionite were added. Aliquots were taken for the reference and sample cuvettes and carbon monoxide was bubbled into the sample cuvette. Spectral determinations were made in a Cary Model 14 spectrophotometer equipped with a 0-0.1 slide wire and a scattered transmission accessory.

Results and Discussion

Preliminary results demonstrated that the 1500 g pellet contains aminopyrine

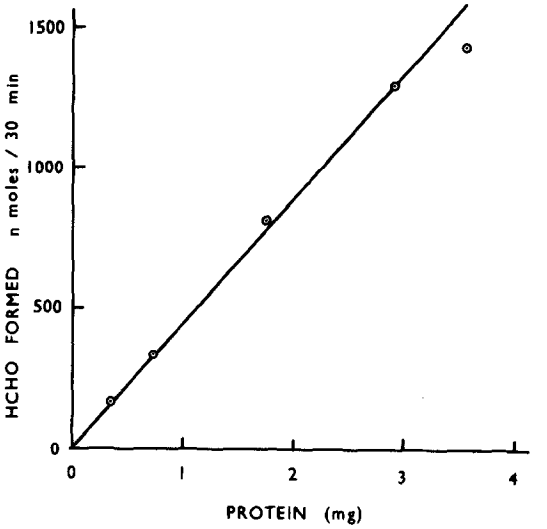

FIG. 1: Aminopyrine Demethylation as a Function of Protein Concentration in the 1,500 g Pellet

Assay and incubation conditions as described in Materials and Methods

demethylating activity. This activity was found to be proportional to protein concentrations up to 3 mg of protein; however activity seems to have levelled off at about 4 mg of protein (Fig. 1). The characteristics of this activity were

TABLE 1

CHARACTERISTICS OF THE MONOOXYGENASE ACTIVITY
- ASSAYED AS AMINOPYRINE DEMETHYLATION -
IN THE 1500 g PELLET

	Incubation Mixture*	% Activity†
Complete		100
"	minus 1,500 g Pellet	4.2
"	minus NADP	2.3
"	minus both G6P and G6PD	0
"	+ SKF 525A (10^{-4}M)	41.0

*Complete incubation mixture represents conditions described in Materials and Methods. G6P = glucose 6-phosphate; G6PD = glucose-6-phosphate dehydrogenase.

†100% activity = 1430 nmoles HCHO formed in 30 min by a complete incubation mixture (3.6 mg of microsomal protein) in an atmosphere of air.

TABLE 2

THE EFFECT OF CARBON MONOXIDE
ON THE AMINOPYRINE DEMETHYLATION BY THE 1500 g PELLET

Gas Phase	% Activity
O_2	100
N_2:O_2 (1:1)	56.2
CO:O_2 (1:1)	29.7

Each incubation contained 3.0 mg of microsomal protein. 100% activity with O_2 as the gas phase, represents a mean of three incubations yielding 723 ± 20 nmoles HCHO/30 min.

TABLE 3

HYDROXYLATION OF Δ^1-TETRAHYDROCANNABINOL (Δ^1-THC) AND DEMETHYLATION OF p-CHLORO-N-METHYLANILINE (PCMA) BY NORMAL MICROSOMES AND BY THE 1,500 g PELLET*

	Demethylation of PCMA	Hydroxylation of Δ^1-THC
Microsomes	p-Chloroaniline formed nmoles/mg protein/15 min	7-OH-Δ^1-THC formed nmoles/mg protein/30 min
Normal	76 ± 7	10.2 ± 1.2
1500 g Pellet	110 ± 10	14.8 ± 1.2

*For conditions of assays see Materials and Methods; there were 4 rats per group. Values represent the mean ± S.E.

found to resemble that of a microsomal monooxygenase (Table 1). Namely, this activity required NADPH generation, whereas $NADP^+$ in the absence of glucose-6-phosphate and glucose-6-phosphate dehydrogenase did not support this monooxygenase activity. Furthermore, SKF 525A [a recognized inhibitor of microsomal monooxygenase (13, 14)], was found to inhibit this enzymatic activity. Furthermore, the requirement for O_2 for maximal activity and the inhibition by carbon monoxide were demonstrated (Table 2). The 1500 g pellet was found also to catalyze the

oxidative demethylation of p-chloro-N-methylaniline (Type II substrate) and the oxidation of Δ^1-THC (Type I substrate) (Table 3). The presence of cytochrome P-450 in the 1500 g pellet was demonstrated; $\Delta O.D_{450-500}$ in one preparation was determined to be 0.07/mg protein/ml.

A characteristic of the hepatic microsomal monooxygenase system is its ability to be induced by barbiturates and chlorinated hydrocarbon insecticides (1, 2). Thus the effect of phenobarbital on the rate of demethylation of aminopyrine by normal and 1500 g microsomes was examined (Table 4). Phenobarbital treatment markedly enhanced the enzymatic activity in both normal and 1500 g preparations.

TABLE 4

EFFECT OF PHENOBARBITAL TREATMENT OF RATS ON AMINOPYRINE DEMETHYLATION BY "NORMAL" AND 1,500 g MICROSOMES

	HCHO formed nmoles/mg Protein/30 min	
Treatment	"Normal" microsomes	1,500 g microsomes
Exp. 1		
Controls	380 ± 63	587 ± 75
Phenobarbital	1053 ± 195*	1236 ± 110**

*P < 0.025; **P < 0.01.

Values represents the mean ± S.E. of 4 rats per group.

Rats were treated with phenobarbital for 3 days b.i.d. (37.5 mg/kg, i.p. each injection); controls received the vehicle (H_2O). Animals were sacrificed 18 hours after last dose. "Normal" and 1500 g microsomes were prepared from the same livers which were evenly divided for the respective preparations.

In conclusion the present studies and those of Kamath *et al* (3) appear to "legitimize" the 1500 g pellet as a functional microsomal fraction.

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