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**PRELIMINARY COMMUNICATION**

# AMINOPYRINE N-DEMETHYLASE ACTIVITY IN NEONATAL RAT SKIN

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## INTRODUCTION

Most studies of microsomal drug-metabolizing enzymes have focused on the liver, although in recent years it has become well established that many extrahepatic tissues also contain cytochrome P-450-dependent microsomal enzyme systems (for an update see reference 1 and chapters therein.). These enzymes are capable of transforming a wide range of endogenous and exogenous substrates. A fundamental premise of pharmacology is that the magnitude and duration of the effects of xenobiotics in a tissue depends upon its concentration in that organ. Since skin is one of the largest extrahepatic tissues and is in continuous contact with numerous xenobiotics, studies to define the scope of drug metabolism in cutaneous tissue, are of great importance. Relatively few studies have been carried out to assess the capacity of the skin to metabolize drugs and chemicals (for an update see 2,3 and references therein). However, it has previously been shown that cutaneous microsomes contain the heme-protein cytochrome P-450 (4-6) and that certain drug metabolizing enzymes, among them, aryl hydrocarbon hydrxylase, 7-ethoxycoumarin O-deethylase, epoxide hydrolase and glutathione-S-transferases are present in cutaneous tissue (2-9).

The substrates for the cytochrome P-450 dependent mixed-function oxidases have been classified as either type I and type II (10). In all published reports on xenobiotic metabolism in skin only type II substrates such as the polycyclic aromatic hydrocarbons have been shown to be metabolized by the cytochrome P-450 system (2-9). We now report that a typical type I substrate, aminopyrine, is also metabolized by microsomes prepared from the skin of neonatal rats. These results indicate that in addition to known P-450 dependent toxication mechanisms, detoxification reactions are also carried out in skin. Additionally, skin cytochrome P-450 is capable of metabolizing a wider class of xenobiotics than heretofore suspected.

## MATERIALS AND METHODS

Animals: Sperm-positive pregnant Sprague-Dawley rats were obtained from the Holtzman Rat Farm, Madison, Wisconsin and shipped during the last trimester. The advantages of using neonatal rat as an experimental model for study on cutaneous drug metabolism have been described earlier (7). Neonatal rats born in situ were allowed to suckle until the 4th day after birth, withdrawn from their mothers, and used in the experiments.

Preparation of Microsomes: The neonatal rats were sacrificed by decapitation with surgical scissors. The head and extremities of the animals were removed and whole skin from the remaining body, which constitutes 80-90% of the total, was excised and immediately placed in ice cold 0.15 M KCl. Each skin was placed epidermal side down on a covered glass petri dish containing crushed ice. The skin was scraped with a sharp scalpel blade (Bard-Parker No. 20) to remove subcutaneous fat and muscle, washed twice with 0.15 M KCl, blotted with paper towel and carefully minced to small pieces ($<1mm^3$).

All subsequent operations were carried out at 4°C. The minced tissue was added to a 50 ml polyethylene beaker, containing 4 volumes of 0.15 M KCl. Each tissue was subjected to six separate bursts of a Polytron Tissue Homogenizer (Brinkman Instruments) equipped with a ST-10 generator. There was a pause of 30 seconds between each burst to permit cooling of the tissue. This whole homogenate was then poured into the tube of a ground glass conical tissue homogenizer fitted with a ground glass pestle made to carefully define tolerances with a clearance 0.004-0.006 mm (Kontes Glass Co., Vineland, N.J.) and was homogenized for six up and down strokes at 400 R.P.M. using a rotary drill press. This whole homogenate was filtered through two layers of surgical gauze soaked in 0.15 M KCl using gentle vacuum with a Buchner funnel. The homogenate was centrifuged at 800xg for 20 minutes in a Sorvall RC 2B refrigerated centrifuge using a SS-24 rotor. The pellet was washed gently and respun at 800xg for 20 minutes. The pooled supernatants were then spun at 9000xg for 20 minutes. The 9000xg pellet was washed and respun at 9000xg for 20 minutes. The pooled 9000xg supernatants were then spun at 100,000xg for 60 minutes in a Beckman L 5-50 ultracentrifuge using a 50 Ti-Rotor. The washed microsomal pellet was overlayed with 0.1 M phosphate buffer pH 7.40 containing 20% glycerol (v/v), 10mM EDTA and 10mM dithiothreitol and was frozen under nitrogen at -170°C until the day of analysis (3-5 days after preparation). Verification of the suitability of these procedures for the preparation of skin, epidermal, and dermal microsomes and the stability of the enzyme activity when stored under these conditions will be reported elsewhere (Bickers et al, In preparation). For comparative studies liver microsomes were made according to conventional techniques described earlier (7).

Assay of N-demethylation of aminopyrine. The highly sensitive radiometric assay of Poland and Nebert (11) was adapted. This procedure allows quantitation of picomolar amounts of ($^{14}C$) formaldehyde. The assay was performed in 20-ml round-bottom centrifuge tubes with a ground-glass stopper. The typical incubation mixture in a final volume of 0.5 ml contained 1 μmol of NADP, 1 μmol of NADH, 20 μmol of glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 2.5 μmol of $MgCl_2$, 20 μmol of potassium phosphate buffer, pH 7.4, 5.0 μmol of semicarbazide and 5.0 μmol of nicotimamide and an aliquot of enzyme source (3-5 mg skin microsomal protein or 0.2-0.3 mg liver microsomal protein) in 100 ul of buffer (100 mM potasium phosphate buffer containing 10 mM dithiothreitol, 10 mM EDTA and glycerol 20% (V/V), pH 7.4). The 20 minute incubation at 37°C was started by the addition of 5.0 μmol of $^{14}C$-aminopyrine (550,000 D.P.M.) in 50 ul of $H_2O$. The reaction was terminated by the addition of 8 ml of cold chloroform and then 1 ml of 0.1 N NaOH was added. Blank tubes contained aminopyrine which was added after incubation for 20 minutes and chloroform was then added immediately. The contents of the tubes were mixed well by vortexing for 2 minutes and the chloroform layer was removed after centrifugation at 1000 xg for 5 min. One more extraction with chloroform was performed, after which a 0.6-ml sample of the aqueous phase was transfered to a scintillation vial and 10 ml of Scintiverse was added and the radioactivity determined in a Searle Mark III Liquid Scintillation Counter with a counting efficiency of 85-90%. The quantity of formaldehyde formed during the incubation is calculated from the net D.P.M. (sample minus blank), corrected to total volume of aqueous phase and recovery of formaldehyde.

Protein Determination. Protein was estimated according to Lowry et al (12) using bovine serum albumin as reference standard.

## RESULTS AND DISCUSSION

The results are summarized in Table 1 and demonstrate the presence of aminopyrine N-demethylase activity in microsomes prepared from the skin of neonatal rats. The drug metabolizing enzyme activity in skin microsomes was NADPH-dependent. Activity in the presence of NADH (1.0 μmol) alone was only 37 percent that of NADPH. Addition of NADH to incubation mixtures containing NADPH resulted in an 8 percent increase in formaldehyde formation. Although this increase was not appreciable we decided to add NADH to routine incubation systems. No formaldehyde formation was noticed when either boiled enzyme (5 minutes in a boiling water bath) was used in the complete incubation system or when the pyridine nucleotide or the regenerating system was omitted from the incubation mixture. Cofactor requirements for neonatal skin aminopyrine-N-demethylation closely resembled those of the hepatic N-demethylase (Table 1).

Table 1

Aminopyrine N-demethylase activity in neonatal rat skin and liver microsomes: Cofactor requirements

| Conditions | p moles HCHO/min/mg protein SKIN | LIVER |
|---|---|---|
| Complete System | 5.9 | 822 |
| Complete System (boiled enzyme) | < 0.2 | < 0.2 |
| Complete System - NADPH generating system | < 0.1 | < 0.1 |
| Complete System - NADPH generating system + NADPH (1.0 μmol) | 6.2 | 803 |
| Complete System - NADPH generating system + NADH (1.0 μmol) | 2.2 | 62 |
| Complete System - NADPH generating system + NADPH (1.0 μmol) + NADH (1.0 μmol) | 6.4 | 842 |

Whole skin microsomal protein (3.4 mg) or liver microsomes (0.26 mg) were incubated for 15 min at 37°C with the components of assay system (1 μmole of NADPH and 1 μmole of NADP + 2 U of G6PDH). This represents the complete system.

Under the conditions of this aminopyrine N-demethylase assay, formaldehyde formation by neonatal rat skin microsomes was directly proportional to the amount of microsomal protein employed (up to 3.8 mg) and to the time of incubation (up to 20 minutes). These data are presented in Figure 1. With incubations longer than 20 minutes, formaldehyde production plateaued (Fig 1A). The deviation from linearity observed during longer incubation periods was not due to depletion of cofactors or substrate as addition of cofactors and/or substrate at this stage did not result in increased formaldehyde formation.

The data presented here clearly show that skin does contain aminopyrine N-demethylase activity and that skin microsomes are active in metabolizing a type I substrate such as aminopyrine. These data

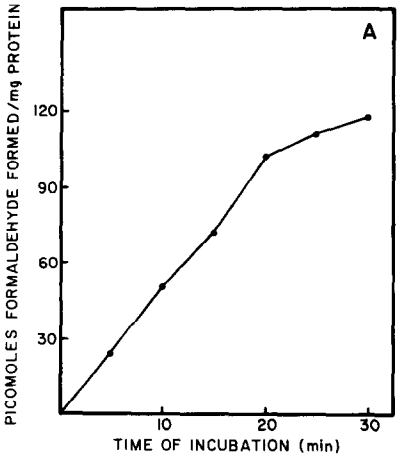


Figure 1A.

Neonatal rat skin microsomal aminopyrine N-demethylase activity as a function of time of incubation. Enzyme activity is presented as picomoles HCHO per mg protein.

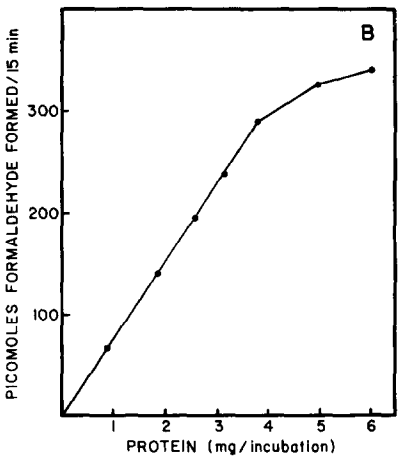


Figure 1B.

Neonatal rat skin microsomal aminopyrine N-demethylase activity as a function of protein concentration. Enzyme activity is presented as picomoles HCHO formed per 15 min.

emphasize that the small amount of measurable P-450 in skin microsomes is capable of considerable metabolism of appropriate substrates. Further studies are underway to better define the range of substrates metabolized by the skin monooxygenase system.

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