A STUDY ON THE PRETHCAMIDE HYDROXYLATION SYSTEM IN RAT HEPATIC MICROSONES

Department of Biochemistry and Drug Metabolism, Medical School, Lubartowska 85, 20-123 Lublin, Poland

Ethyl butamide and propyl butamide, the active constituents of the analeptic drug named Prethcamide (Ciba-Geigy), undergo biotransformation to respective single metabolites in the presence of rat hepatic microsomes and the NADPH-generating system. Spectral analysis showed that the metabolites were hydroxylated forms of the drug. The hydroxylation was stimulated by NADH and increased ionic strength, and inhibited by the known cytochrome P-450 inhibitors, e.g. SKF-525A, metyrapone, CO and KCN. The drug formed type I binding spectrum with cytochrome P-450.

It has been well documented and is widely accepted that the hepatic microsomal mixed function oxidase system plays a central role in the metabolism (mainly hydroxylation) of various endogenous substrates such as steroids, bile acids and fatty acids and also numerous exogenous substances (xenobiotics) such as drugs, pesticides, carcinogens etc. (Orrenius & Ernster, 1974; Paine, 1981). The major component of the system is cytochrome P-450 that functions as a terminal oxidase and binds to the substrates producing characteristic spectral changes of various types (Schenkman et al., 1967). Recent studies have revealed that cytochrome P-450 is actually a family of isoenzymes which possess overlapping substrate specificity and properties (Guengerich, 1979). Some of the cytochromes are differentially induced by drugs and other organic compounds and some are involved in biotransformation of compounds to be eliminated. To understand the multiplicity of cytochromes P-450 and the mechanism of their induction is an important problem. In search for a new convenient model of drug biotransformation to be used for induction studies on different species, we have chosen the Prethcamide hepatic hydroxylation system.

Prethcamide (Micoren), an analeptic drug, is a mixture of equal parts of two derivatives of crotonyl-butyric acid: N-crotonyl-2-ethyaminobutyric acid dimethylamide (ethyl butamide) and N-crotonyl-2-propylaminobutyric acid dimethylamide (propyl butamide). Clinical use of Prethcamide has been extensive (Ref. in Geigy leaflet: Micoren-Respirotonico, 1976) but no information is available on pharma-
kinetic characteristics and metabolism of the drug. Our preliminary studies with rats have shown that the drug is quickly eliminated from the body; its low bioavailability (about 20%) points to the appearance of the so-called "first pass effect" and intense metabolism in liver.

In the present work we have attempted to characterize the Prethcamide biotransformation system in rat hepatic microsomes, to elucidate the role played by cytochrome P-450 and to identify the metabolites formed.

MATERIALS AND METHODS

Preparation of liver microsomes. Hepatic microsomes from male Charles River CD rats weighing 200 - 250 g were prepared by differential centrifugation. The animals were killed by decapitation and the livers were immediately excised and homogenized with four volumes of ice-cold 30 mm-phosphate buffer, pH 7.4, containing 1.15% KCl, with the use of a Teflon-glass homogenizer. The homogenate was centrifuged at 9000 g for 20 min and the resulting supernatant was centrifuged again at 105 000 g for 60 min. The microsomal pellet was suspended in the phosphate-KCl buffer to a protein concentration of about 10 mg/ml. Protein was determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

Reagents. Prethcamide was from Ciba-Geigy Ltd (Switzerland); NADP, NADH, glucose 6-phosphate and yeast glucose-6-phosphate dehydrogenase from Behringer (F.R.G.); SKF-525A (2-diethylaminomethyl-2,2-diphenylvalerate) and metyrapone (2-methyl-1,2-bis(3-pyridyl)-1-propanone) were from Smith, Klein and French Lab. (U.S.A.); OV-17, 3%, on Gas-Chrom Q and BSTFA (N,O-bis-trifluoracetamide) from Applied Science (U.S.A.); Amberlite XAD-2 from Rohm and Haas (U.S.A.); silica-gel F-254 plates from Merck (F.R.G.).

Assay. Unless otherwise stated, each incubation mixture contained in 5 ml: phosphate buffer, pH 7.0, 200 μmol; NADP, 1.5 μmol; glucose 6-phosphate, 50 μmol; glucose-6-phosphate dehydrogenase, 0.5 I.U.; ethyl butamide or propyl butamide, 2.5 μmol; and the microsomal fraction, 10 - 12 mg protein. The incubation was performed in a Dubnoff metabolic shaker at 37°C under air. The NADPH-generating system was incubated separately for 15 min at 37°C before the addition of other constituents. The inhibitors were not preincubated with the microsomal fraction.

The reaction was stopped, usually after 5 min, by adding 1 ml of 15% ZnSO₄ followed by 1 ml of saturated BaCO₃ solution. The suspension was stirred and centrifuged for 5 min at 2000 g. The supernatant was passed through commercial 1 g-prepacked Amberlite XAD-2 column. After washing the column with deionized water (about 20 ml), the metabolites and remaining substrates were eluted from the column with 15 ml of methanol. The solvent was evaporated in a rotary evaporator at 40°C, the residue was dissolved in acetone and used for gas chromatography.
Gas chromatography analysis. This was carried out using a Fractovap Model G apparatus (Carlo Erba, Italy) or a Chromatron Model GCHF 18.3-4 (G.D.R.), both equipped with FID detectors. The stationary phase was OV-17, 3%, on Gas-Chrom G (100 - 120 mesh) packed into a glass column (2 m long, 4 mm internal diameter). The oven temperature was 200°C and injection port temperature 230°C. The flow rates were: nitrogen (carrier gas), 54 cm³/min; air, 340 cm³/min; hydrogen, 45 cm³/min.

Nonadecane (19C-hydrocarbon) was used as an internal standard (20 ng/µl in acetone) for quantitative determinations. The metabolites were analysed either as such or silylated with BSTFA. The latter procedure was performed with the flushing technique, by injection of a mixture of 1 µl of the sample and 1 µl of BSTFA onto the column. These conditions assured stoichiometry of the reaction. The amount of Prethcamide components was calculated from the calibration curve of the standards, and the amount of metabolites from the curve of propyl butamide.

Thin-layer chromatography. Standards and reaction products were applied on silica-gel G-60 plates containing fluoresceine and were subjected to chromatographic separation in two solvent systems: I, isomyl alcohol, acetic acid, water (40:10:50, by vol.), and II, ethyl acetate, n-butanol, acetic acid (60:35:5, by vol.). Spots were detected under u.v. lamp or visualized by exposition to iodine vapour. For preparative chromatography, samples were applied in the form of zones, and the separated bands were eluted with acetone.

Isolation of propyl butamide metabolite. The incubation mixture contained in 250 ml: propyl butamide, 12 mg; NADP, 48 mg; glucose 6-phosphate, 600 mg; MgCl₂, 200 mg; glucose-6-phosphate dehydrogenase, 400 I.U.; microsomal fraction equivalent to 20 g of liver, and 10 mM-phosphate buffer, pH 7.0. After incubation for 3 h at 37°C the mixture was centrifuged and the supernatant passed through Amberlite XAD-2 column (20 × 1.5 cm) in a cold-room. The column was washed with 100 ml of water and the metabolites and the remaining propyl butamide were eluted with 100 ml methanol. The eluate was concentrated to dryness, the residue dissolved in 20 ml acetone and chromatographed in solvent system II. The bands of the metabolite were scraped off and the metabolite was eluted with 15 ml methanol. The solvent was evaporated and the residue suspended in 5 ml acetone. After centrifugation, acetone was evaporated and the oily residue (about 6 mg), similar in consistence to the substrate, was used for chromatographic and spectral analyses. The material gave a single peak in gas chromatography and a single spot on thin-layer chromatography.

Spectral analysis of substrates and metabolites. Spectrophotometric analysis revealed a sharp peak of absorbance at 274 nm, common for substrates and metabolites.

Gas chromatography - mass spectrometry. The combination instrument was an LKB 9000 operated at an accelerating voltage of 3.5 kV, ionization voltage of 70 eV
and trap current 60 μA. Ion source temperature was 290°C. Samples were introduced by gas chromatography under the conditions described above.

Paramagnetic resonance (PMR) spectra of propyl butamide metabolite were recorded at 270 MHz on a Varian XL-100 spectrometer with tetramethylsilane (TMS) in CDCl₃ as an internal reference.

Both components of the drug, their respective metabolites and metabolites treated with BSTFA were subjected to analysis by GC-MS. The data (not shown) revealed that the metabolites were hydroxylated forms of the drug components, having an -OH group at the crotonyl moiety. When treated with BSTFA, hydroxyl was blocked by trimethylsilyl radical. In accordance with these findings were the results of PMR analysis (not shown) which was performed with propyl butamide only. Again, a hydroxyl at the crotonyl moiety was observed.

RESULTS AND DISCUSSION

Incubation of Prethcamide with rat hepatic microsomes and the NADPH-generating system resulted in the formation of a single metabolite from each constituent of the drug, as analysed by gas chromatography (Fig. 1). The metabolites were

---

Fig. 1. Gas chromatography analysis of the microsomal incubation mixture with Prethcamide components. a, Control; b, microsomes incubated with ethyl butamide (EB); c, microsomes incubated with propyl butamide (PB); a', b', c', as before but silylated with BSTFA; M, respective metabolites of EB and PB.
subjected to silylation to more volatile derivatives (shorter retention times). The formation of metabolites was confirmed by thin-layer chromatography. The $R_f$ values of the substrates and metabolites in solvent system I were: ethyl butamidine, 0.60; its metabolite, 0.45; propyl butamidine, 0.65; its metabolite, 0.52.

The metabolites could be extracted with polar solvents, e.g., ethyl acetate or diethyl ether, or adsorbed on the non-ionic resin Amberlite XAD-2. However, the solvent extraction method proved to be rather inefficient as several extractions were needed to achieve a satisfactory recovery. On the other hand, the metabolites were quantitatively adsorbed on the resin under conditions described and could be readily eluted with the solvent system used. The capacity of the analytical 1 g-resin column was at least 12 ml of incubation mixture and the amount of the metabolites recovered was linearly related to the volume of the incubation mixture applied.

From the spectral analysis of the isolated metabolites and their silylated derivatives (data not shown) it appeared that Prethcamide components were hydroxylated at the crotonyl moiety, and then silylated to trimethylsilyl derivatives as presented in Scheme 1.

```
CH₃CH₂CHCON(CH₃)₂
CH₃(CH₂)n NCOCH = CHCH₃
\[\text{cut P-450}\]
CH₃CH₂CHCON(CH₃)₂
CH₃(CH₂)n NCOCH = CHCH₂OH
\[\text{BSTFA}\]
CH₃CH₂CHCON(CH₃)₂
CH₃(CH₂)n NCOCH = CHCH₂OSi(CH₃)
\[n=1, \text{ ethyl butamidine}\]
\[n=2, \text{ propyl butamidine}\]
```

Scheme 1

Further experiments established the requirements for Prethcamide hydroxylation and pointed to the participation of cytochrome P-450. The enzyme activity was found to be located in the microsomal fraction (Table 1), and the 105 000 g supernatant proved to be entirely inactive. The reaction was NADPH- and oxygen-dependent. Moreover, a requirement for magnesium ions was also observed. The residual activity found in the atmosphere of nitrogen can be considered to result from the ineffectiveness of oxygen evacuation from the incubation mixture. On the other hand, a substantial activity of the system observed on omission of glucose-6-phosphate dehydrogenase can be accounted for by possible contamination with this enzyme derived from the cytoplasm.

The role of NADPH as the donor of reducing equivalents for oxidation of drugs and other xenobiotics in liver microsomes has been thoroughly documented (Gillette, 1966; Estabrook et al., 1970). NADH is a poor substitute for NADPH in these
Table 1

Cofactor requirements for Prethcamide hydroxylase

The hydroxylase activity in the complete system was measured under standard assay conditions.

The activity is expressed in nmol/min per mg protein.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Ethyl butamide</th>
<th>Propyl butamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity</td>
<td>%</td>
</tr>
<tr>
<td>Complete system (control)</td>
<td>2.45</td>
<td>100</td>
</tr>
<tr>
<td>Microsomes omitted</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Microsomes omitted, 105 000 g supernatant added</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NADP omitted</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose 6-phosphate omitted</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase omitted</td>
<td>1.61</td>
<td>66</td>
</tr>
<tr>
<td>Oxygen omitted*</td>
<td>1.09</td>
<td>45</td>
</tr>
<tr>
<td>MgCl₂ omitted</td>
<td>2.07</td>
<td>84</td>
</tr>
<tr>
<td>NADP omitted, 2.5 μmol NADH added</td>
<td>0.77</td>
<td>32</td>
</tr>
<tr>
<td>Complete system, 2.5 μmol NADH added</td>
<td>4.72</td>
<td>192</td>
</tr>
</tbody>
</table>

* The mixture was bubbled with N₂ for 5 min, then the tube was stoppered and incubated without shaking.

reactions; it supports the reaction rate to only 10 - 15% of that with NADPH. However, NADH stimulates the NADPH-dependent reaction by 30 - 100% (Conney et al., 1957; Cohen & Estabrook, 1971; Gillette, 1971; Correira & Mannering, 1973a,b; Netter & Jilling, 1974). In the present investigation we obtained a slightly higher activity of hydroxylation with NADH alone, amounting to approximately 30%, and stimulation of the NADPH-dependent reaction by about 90%. We believe that the latter phenomenon can be classified as the well-known "NADH synergism", in which NADH is considered to be a supplier of the second electron via cytochrome b₅ for the reduction of the oxygenated reduced cytochrome P-450 substrate complex (Mannering, 1975).

The time-course of the enzymatic reaction was followed by incubating Prethcamide components with microsomes during 30 min and measuring the amount of the metabolites formed. The reaction exhibited linearity up to 10 min for ethyl butamide and 7.5 min for propyl butamide (Fig. 2). The rate of hydroxylation also revealed a linear dependence on concentration of protein from 3 to 18 mg per 5 ml of the incubation mixture. In the routine kinetic determinations the incubation time was 5 min, and protein 10 - 12 mg.

The dependence of the hydroxylation rate on substrate concentration was measured by incubating the microsomal fraction with ethyl butamide (Fig. 3) and propyl butamide in the range of 0.1 - 5 mM. The respective Kₘ values calculated from the Lineweaver-Burk plots were 0.20 and 0.22 mM, and Vₘₙₐₓ values were 2.85 and 3.12 nmol/min per mg protein.

The pH optimum of the reaction was between 6.7 and 7.3 (not shown).
The Prethcamide hydroxylating system was activated by potassium chloride at the concentration of 2 - 10 mM by 40 - 60% both with ethyl butamide and propyl butamide. A further increase in the salt concentration up to 40 mM caused a slight inhibition. This phenomenon should be most likely considered in terms of increased ionic strength since higher concentrations of phosphate buffer also decreased the reaction rate. Increasing ionic strength has also been shown to enhance cytochrome P-450-mediated NADPH- and NADH-dependent peroxidase reactions (Hrycay & O’Brien, 1974).

SKF-525A, a typical cytochrome P-450 inhibitor (Anders & Mannering, 1966) lowered the rate of hydroxylation of Prethcamide components by about 90% when used at a concentration of 20 μM (Table 2), whereas at a concentration as low as 5 μM...


**Table 2**

*Effect of inhibitors on Prethcamide hydroxylase activity*

The hydroxylase activity was assayed under standard conditions. The activity is expressed in nmol/min per mg protein. The inhibitors were added without preincubation.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Ethyl butamide</th>
<th>Propyl butamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity</td>
<td>Activity</td>
</tr>
<tr>
<td>None</td>
<td>2.36</td>
<td>1.97</td>
</tr>
<tr>
<td>SKF-525A (20 µM)</td>
<td>0.39</td>
<td>0.43</td>
</tr>
<tr>
<td>Metyrapone (0.2 mM)</td>
<td>1.36</td>
<td>1.20</td>
</tr>
<tr>
<td>t-Amyl alcohol (7%)</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>Acetone (5%)</td>
<td>1.19</td>
<td>0.96</td>
</tr>
<tr>
<td>Carbon monoxide*</td>
<td>0.32</td>
<td>0.44</td>
</tr>
</tbody>
</table>

* The mixture was bubbled with CO for 10 min in an ice-bath.

the inhibition still amounted to 50%. Thus, the effectiveness of inhibition by SKF-525A was about ten times higher than in other systems described (Kato, 1979). Metyrapone, another potent cytochrome P-450 inhibitor (Mitani et al., 1982), lowered the reaction rate by 40% at a concentration of 0.2 mM. Acetone and tertiary amyl alcohol, the reagents that affect the membrane structure of microsomes and convert cytochrome P-450 to its inactive P-420 form (Hrycay & O’Brien, 1974), were also efficient inhibitors of Prethcamide hydroxylation. In addition, carbon monoxide, which is a characteristic cytochrome P-450 blocker (Omura & Sato, 1964), inhibited the reaction considerably. Finally, cyanide that is capable of serving as a modified type II ligand to hepatic microsomal cytochrome P-450 (Hrycay & O’Brien, 1974), acted as an effective inhibitor, causing a 50% decrease of the reaction rate at the concentration of 0.5 mM (Fig. 4). This indicates that a cytochrome P-450 species of higher affinity for cyanide is involved in hydroxylation of Prethcamide.

![Fig. 4. Effect of KCN on the rate of Prethcamide hydroxylation.](image-url)
The hydroxylating system was heat-labile (Fig. 5). Incubation for 1 min of the microsomal suspension at 55°C resulted in about 90% loss of the activity. On the other hand, the system was quite stable at 37°C, being inactivated only by 8% during two hours. Table 3 shows the effect of temperature on the rate of Prethcamide hydroxylation. The $Q_{10}$ coefficient of about 2 is typical for most of enzyme reactions. The activation energy is slightly lower for propyl butamide than for ethyl butamide.

**Table 3**

*Effect of temperature on the rate of Prethcamide hydroxylation*

NADPH (2.5 μmol) was substituted for NADP. The activity is expressed in nmol/min per mg protein and activation energy in cal/mol. Incubation lasted 5 min.

<table>
<thead>
<tr>
<th></th>
<th>Ethyl butamide</th>
<th>Propyl butamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity at 27°C</td>
<td>1.44</td>
<td>1.28</td>
</tr>
<tr>
<td>Activity at 37°C</td>
<td>3.07</td>
<td>2.43</td>
</tr>
<tr>
<td>$Q_{10}$</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Activation energy</td>
<td>13 900</td>
<td>11 900</td>
</tr>
</tbody>
</table>

It seems worthwhile to add that the cytochrome P-450-Prethcamide complex exhibits type I binding spectrum, with the trough at 419 nm and the peak at 368 nm (not shown). This is in agreement with the earlier mentioned occurrence of "NADH synergism" in Prethcamide hydroxylation. According to Correira & Mannering
(1973a) and Netter & Jilling (1974) only the metabolism of type I substrates is synergized by NADH. This provides evidence that the entry of the second electron for the reduction of the ternary reduced cytochrome P-450 - molecular oxygen - substrate complex is rate limiting.

One of us (T.S.) is much indebted to Professor Silvio Garattini for the possibility of staying at the Istituto di Ricerche Farmacologiche "Mario Negri" in Milan, Italy, where a part of these studies was performed.

REFERENCES


Correia, M.A. & Mannering, G.J. (1973a) DPNH synergism of TPNH-dependent mixed function oxidase reactions. *Drug Metab. Disp.*, 1, 139-149.


BADANIA NAD SYSTEMEM HYDROKSYLACJI PRETHCAMIDU
W MIKROSONACH WĄTROBY SZCZURA

Stręszczenie

Etylbutamid i propylibutamid, aktywne składniki leku analeptycznego Prethcamidu, ulegają
przemianie do hydroksylowych pochodnych w mieszaninie inkubacyjnej zawierającej frakcję mikro-
sonalną wątrob szczura i układ wytwarzający NADPH. Hydroksylacja była stymulowana przez
NADH i podwyższoną siłę jonową, a hamowana przez znane inhibitory cytochromu P-450, tj.
SKF-525A, metyrapon, tlenek węgla i KCN. Składniki Prethcamidu tworzyły typ I spektrum wiąza-
nia z cytochromem P-450.

Received 30 May, 1984,
revised 27 August, 1984