# COMUTAGENESIS-II
# SOME FACTORS INFLUENCING THE *IN VITRO* METABOLISM OF 2-AMINO-3-METHYLPYRIDINE

T. Gul Altuntas[1] and J.W. Gorrod[2*]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ankara, 06100 - Tandogan - Ankara, Turkey*
[2]*Chelsea Department of Pharmacy, King's College London, University of London, Manresa Road, London SW3 6LX, UK*

**SUMMARY**

Factors affecting the metabolism of 2-amino-3-methylpyridine (2A3MP) *in vitro* have been studied and the conditions which allow maximal metabolism established. Ring nuclear and methyl hydroxylation, and 1-N-oxidation of 2A3MP were linear with respect to arochlor 1254 induced rat S9 supernatant (10,000 *g* fraction) up to 4.86 mg per ml. The results showed that 20 min incubation time was adequate to observe metabolites formed from 2A3MP. The rate of metabolite production increased with increase in substrate concentration up to 2 µmol per incubate. Using the data obtained the apparent $K_m$ and $V_{max}$ values were calculated using Hanes-Wolf and Lineweaver-Burk plot. No N-hydroxylation of the *exo*-amino group was observed.

**KEY WORDS**

2-amino-3-methylpyridine, nuclear and methyl C-hydroxylation, N-oxidation, *in vitro* metabolism, incubation time, substrate concentration, protein concentration, kinetic constants ($K_m$ , $V_{max}$)

* Author for correspondence

## INTRODUCTION

2-Amino-3-methylpyridine (2A3MP) is known to be mutagenic towards *Salmonella typhimurium* TA98 only in the presence of norharman and a metabolic activating system (S9) /1/. In order to investigate the mechanism of comutagenesis we have first examined the metabolism of 2A3MP using fortified hepatic fractions from the rat and rabbit /2/. It was shown that 2A3MP was metabolised by both species to yield 2-amino-3-methylpyridine-1-N-oxide (2A3MPNO), 2-amino-3-hydroxymethylpyridine (2A3HMP) and 2-amino-3-methyl-5-hydroxypyridine (2A3M5HP). Metabolic products having the characteristics of 2-hydroxylamino-3-methylpyridine or the corresponding nitroso or nitro compounds have not been conclusively identified /2/. As the failure to detect metabolites derived via oxidation of the *exo*-amino group in earlier experiments /2/ may have been due to the enzyme source, we carried out incubations using a S9 hepatic fraction derived from arochlor 1254 induced rats in order to mimic conditions usually used to examine compounds for mutagenesis or comutagenesis. We now report the effects of various factors on the C-hydroxylation and N-oxidation of 2A3MP using arochlor 1254 induced rat hepatic S9 fraction as enzyme source.

## MATERIALS AND METHODS

2-Amino-3-methylpyridine (2A3MP) and 3-hydroxypyridine were purchased from Aldrich Chemical Co. (Gillingham, Kent, UK). Glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were supplied by Sigma Chemical Co. (Poole, Dorset, UK). Glucose-6-phosphate dehydrogenase suspension grade II from yeast (140 U/mg) was obtained from Boehringer Mannheim Co. (Lewes, Sussex, UK). Arochlor 1254 was obtained from Eli Lilly and Co. (Indianapolis, IN, USA) as a gift. All other solvents and chemicals were of the highest commercially available grade from British Drug Houses Ltd. (Poole, Dorset, UK).

Male Albino Wistar rats (200-300 g) were supplied from King's College London Biological Services Facility.

**Preparation of potential metabolites of 2A3MP**

All metabolites of 2A3MP were prepared as described previously /2/.

**Pretreatment of animals**

Pretreatment of rats was carried out using arochlor 1254 (500 mg/kg i.p. in 0.5 ml corn oil) given as a single dose, animals being sacrificed on the fourth day. The animals were deprived of food the night before tissue preparation, but were given drinking water *ad libitum*.

**Preparation of hepatic S9 supernatant (10,000 *g* fraction)**

Hepatic S9 supernatants from rats were prepared by the method of Gorrod *et al.* /3/. The supernatants were either used fresh on the day of preparation, or stored at -80°C until required. Protein concentration in the supernatant was determined using Folin and Ciocalteau's phenol reagent, essentially as described by Lowry *et al.* /4/.

**Incubation of 2-amino-3-methylpyridine**

Cofactor solution consisted of $NADP^+$ (2 µmol), glucose-6-phosphate (10 µmol), magnesium chloride (20 µmol) in phosphate buffer (2 ml, 0.2 M, pH 7.4). Pre-incubation of cofactor solution was carried out in a shaking water bath at 37°C for 5 min before the addition of hepatic S9 supernatant (1 ml) and 2-amino-3-methypyridine (2A3MP). Reactions were terminated by placing flasks on ice, when the internal standard (3-hydroxypyridine, 20 nmol in 20 µl water) was added. The substrate and its metabolites were immediately extracted with a mixture of chloroform (4 ml) plus ethanol (1 ml) followed by chloroform (3x4 ml) using a rocking apparatus for 15 min. The organic layers were bulked and evaporated to dryness using a stream of nitrogen at room temperature. The residues were reconstituted in mobile phase (100 µl) prior to HPLC analysis.

In certain experiments the incubation time or the concentration of substrate or amount of enzyme preparation was varied.

**HPLC analysis**

The metabolic incubates were analysed by HPLC by injecting 20 µl of concentrated metabolic extract into the HPLC system. HPLC was performed on a Nucleosil 5 µm SA analytical column (25 cm length, 4.6 mm i.d.; Fisons Chromatography, Leics, UK) and a guard column. The HPLC system consisted of two Altex (model 110A and 110B) solvent delivery systems coupled to a NEC PC 8300 gradient controller and a Rheodyne syringe loading sample injector valve (model 7125) fitted with 20 µl sample loop (Thames Chromatography, Berks, UK). The UV detection was performed at 290 nm with a Philips Pye Scientific Roseate Chromatography data analysis system (version 4.0). The solvents used for chromatography consisted of 0.05M $NH_4H_2PO_4$ (solvent A) and 0.1M $NH_4H_2PO_4$ containing 50% v/v methanol (solvent B).

The elution of 2A3MP and its metabolites was performed using the computer gradient programme to give a flow rate of 1.5 ml/min. The gradient system used is as follows: At zero time solvent A=100%; from zero to 10 min, solvent B was increased to 50%, and from 10 min to 28 min, solvent B was increased to 100% and held for 15 min before returning to A=100%.

**Quantitative determination of 2A3MP metabolites**

Calibration curves for the quantification of 2A3MP metabolites were prepared. Various amounts of authentic metabolites (10-1000 pmol) and internal standard (3-hydroxypyridine, 20 nmol) were added to inactivated hepatic S9 mixtures in duplicate. The extraction and HPLC analysis of substrate and metabolites were performed as described under incubation procedure. A calibration curve for each compound was constructed by plotting the ratio of area of the peak produced by the compound to that of internal standard against known amounts of the compound added.

**Effect of varying protein concentration**

Incubations were performed using various concentrations of resuspended S9 supernatant from arochlor 1254 induced rats (1 ml containing varying concentrations of S9 supernatant) using 2 µmol of 2A3MP at 37°C with standard cofactor solution (2 ml in phosphate buffer, 0.2

M, pH 7.4). The C-hydroxylated and N-oxidised products formed metabolically were quantified using the HPLC analytical method described above.

**Effect of varying incubation time**

Incubates were prepared containing cofactor solution (2 ml), 2A3MP (2 μmol in 50 μl water) and S9 supernatant from arochlor 1254 induced rats (equivalent to 0.4 g original liver weight per ml). Incubations were carried out for various times (0-60 min) at 37°C and metabolically produced compounds were quantified as above.

**Effect of varying substrate concentration**

The incubation mixture consisted of cofactor solution (2 ml), S9 supernatant (1 ml) and varying concentrations of 2A3MP (0.1-10 μmol/3 ml). Incubations were carried out for 20 min at 37°C and the metabolites quantified using the HPLC method above.

**Kinetic properties of C- and N-oxidation of 2A3MP**

The steady state kinetics of arochlor 1254 induced rat liver P450 enzyme with 2A3MP were studied using the data obtained by variation of 2A3MP concentration. The apparent $K_m$ and $V_{max}$ values for 2A3MP metabolites were determined according to the direct linear-plot method (Lineweaver-Burk plot and Hanes-Wolf plot).

## RESULTS AND DISCUSSION

Using the gradient HPLC conditions, the retention time ($R_t$) values for 2A3MP and its oxidation products were well separated (Figure 1) and the $R_t$ values are given in Table 1.

The chromatograms clearly show the presence of 2A3MPNO, 2A3HMP and 2A3M5HP; these metabolites were further characterised by comparing their UV spectra with authentic compounds using a Rapiscan diode array detector as described earlier /2/. Again no evidence was obtained for the formation of 2-hydroxylamino-3-methyl-pyridine (2H3MP), 2-nitroso-3-methylpyridine (2NO3MP) or 2-nitro-3-methylpyridine (2N3MP), or their azo or azoxy condensation products.

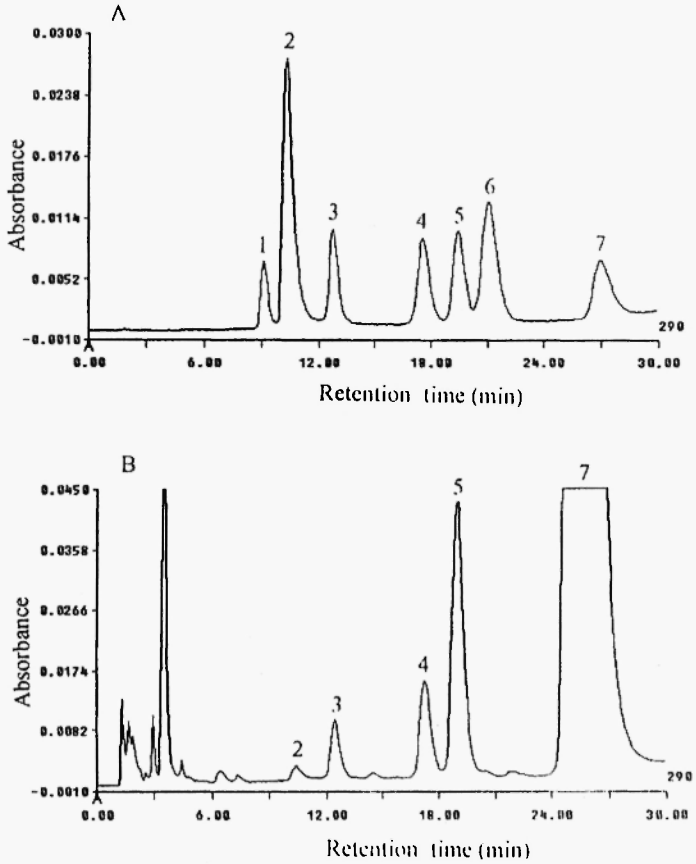


**Fig. 1:** HPLC chromatogram of (A) authentic standards, and (B) metabolic extracts, when 2A3MP was incubated with arochlor 1254 pretreated rat S9 fractions under optimal conditions. (See text for abbreviations.) (1) 2N3MP; (2) 2A3MPNO; (3) Internal standard (3-hydroxypyridine); (4) 2A3HMP; (5) 2A3M5HP; (6) 2H3MP; (7) 2A3MP.

The effects of variation of protein concentration, incubation time and substrate concentration on the *in vitro* metabolism of 2A3MP are shown in Figures 2, 3 and 4, respectively. A linear relationship was established between all routes of oxidative metabolism and hepatic protein concentration up to 0.4 g wet weight of liver/3 ml of incubate (4.86 mg per ml) using arochlor 1254 induced rat S9 supernatant

**TABLE 1**

HPLC separation of 2A3MP from its possible metabolites

| Compounds | | Retention time (min) |
|---|---|---|
| 2A3MP | 2-amino-3-methylpyridine | 27.0 |
| 2N3MP | 2-nitro-3-methylpyridine | 9.1 |
| 2A3MPNO | 2-amino-3-methylpyridine-1-N-oxide | 10.3 |
| 2A3HMP | 2-amino-3-hydroxymethylpyridine | 17.3 |
| 2A3M5HP | 2-amino-3-methyl-5-hydroxypyridine | 19.0 |
| 2H3MP | 2-hydroxylamino-3-methylpyridine | 21.1 |
| 2NO3MP | 2-nitroso-3-methylpyridine | 10.0 |

Apparatus and stationary phase as described in text.
Mobile phase, (A) $NH_4H_2PO_4$ (0.05 M) (B) $NH_4H_2PO_4$ (0.1 M) containing 50% v/v methanol; flow rate, 1.5 ml/min; detection wavelength, 290 nm.

(Figure 2). Increasing the protein concentration beyond this value caused the formation of all three metabolites to decline. Therefore all further incubations were carried out using S9 preparation equivalent to 0.4 g original liver weight per incubate.

Ring and methyl hydroxylation and N-oxidation of 2A3MP were approximately linear up to 20 min incubation using arochlor 1254 induced rat S9 supernatant (Figure 3), after which the rate of production of metabolites declined to different extents.

The effect of substrate concentration on the *in vitro* metabolism of 2A3MP (Figure 4) showed that 1-N-oxidation of 2A3MP increased approximately linearly up to 2 μmol per 3 ml incubate. Beyond this

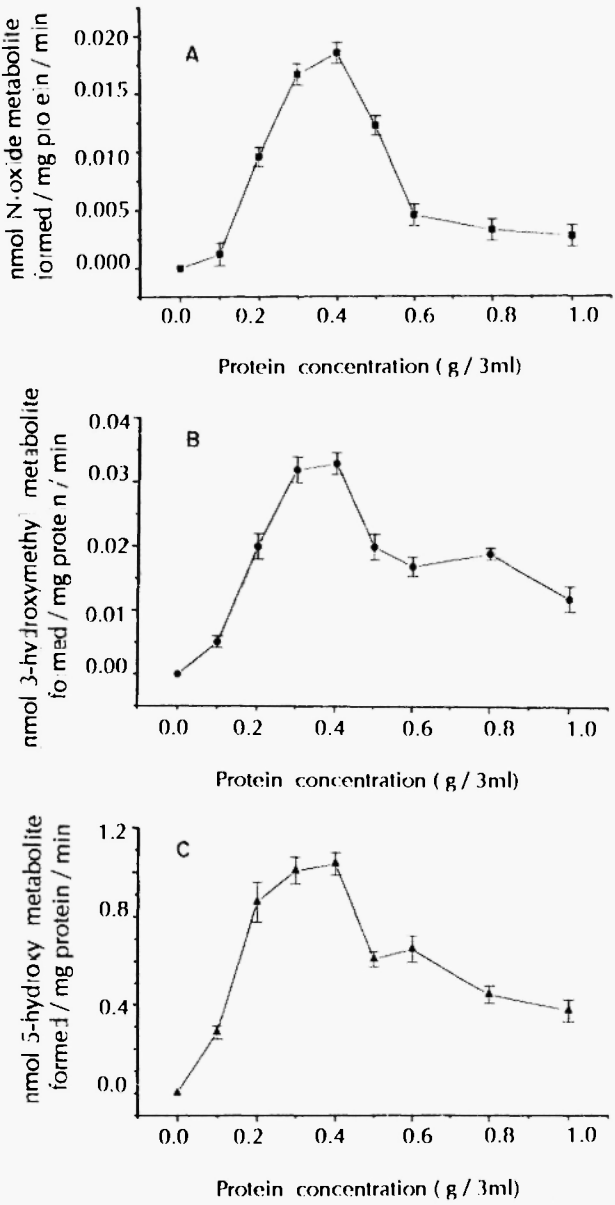


**Fig. 2:** The effect of hepatic protein concentration on the formation of 2A3MPNO (A), 2A3HMP (B) and 2A3M5HP (C) using male arochlor 1254 pretreated rat S9 supernatants. The results are means ± SD of duplicate determinations from four animals.

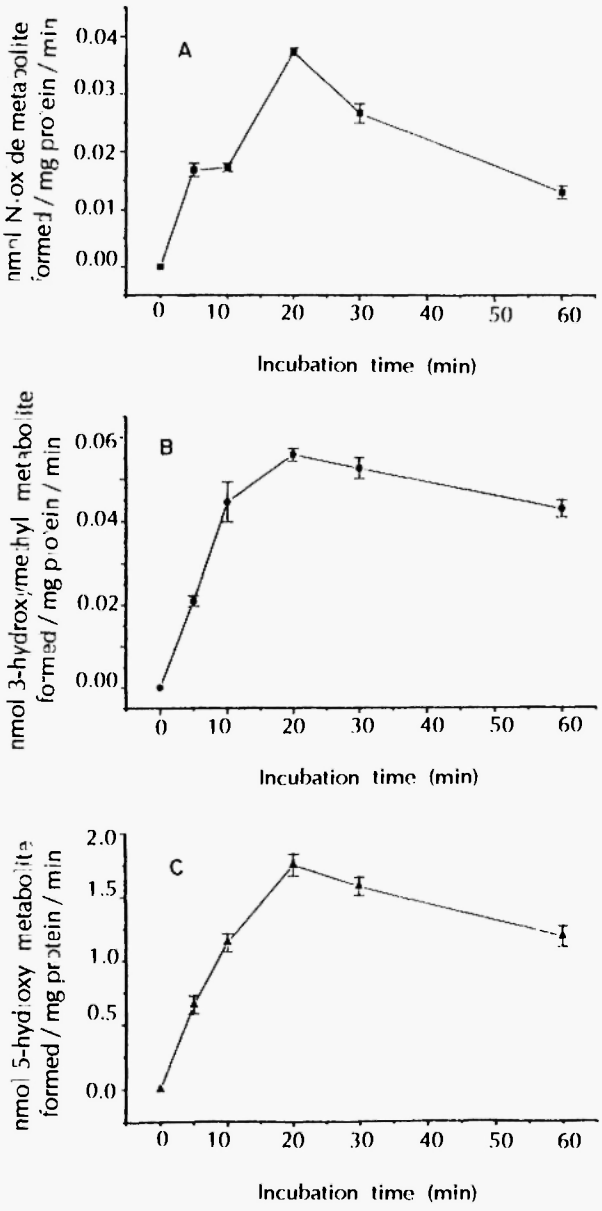


**Fig. 3:** The effect of incubation time on the formation of 2A3MPNO (A), 2A3HMP (B) and 2A3M5HP (C) using male arochlor 1254 pretreated rat S9 supernatants. The results are means ± SD of duplicate determinations from four animals.

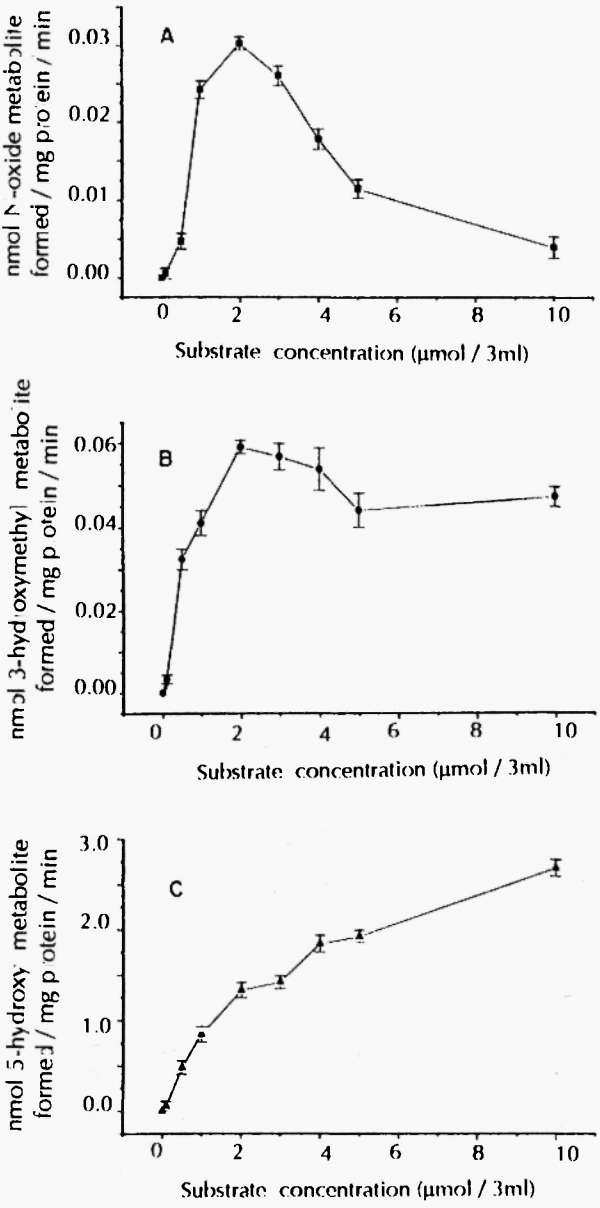


**Fig. 4:** The effect of substrate concentration on the formation of 2A3MPNO (A), 2A3HMP (B) and 2A3M5HP (C) using male arochlor 1254 pretreated rat S9 supernatants. The results are means ± SD of duplicate determinations from four animals.

concentration there was a marked decrease (Figure 4A). Similarly the metabolic formation of 2A3HMP was linear up to 2 µmol per flask, after which any increase in the concentration of 2A3MP decreased the production of 2A3HMP (Figure 4B). The effect of substrate concentration on the metabolic formation of 2A3M5HP also showed linearity up to about 2 µmol per flask after which the amount of 2A3M5HP formed continued to rise slightly over the concentration range used (Figure 4C). These results were used in the determination of kinetic constants (apparent $K_m$ and $V_{max}$) (Table 2).

In a previous publication /2/ we showed that the *in vitro* metabolism of 2A3MP by untreated rat and rabbit hepatic microsomes and S9 fraction produced three primary metabolites, viz. the 1-N-oxide, 2-amino-3-hydroxymethylpyridine and 2-amino-3-methyl-5-hydroxypyr-

**TABLE 2**

Enzyme kinetic constants ($K_m$ and $V_{max}$) for the arochlor 1254 induced rat hepatic S9 supernatant on the C- and N-oxidation of 2A3MP

| Plot | | 2A3MPNO | 2A3HMP | 2A3M5HP |
|---|---|---|---|---|
| HW | $K_m$ | 1.53 | 1.01 | 3.06 |
| | $V_{max}$ | 0.04 | 0.05 | 3.92 |
| LB | $K_m$ | 1.73 | 1.83 | 3.44 |
| | $V_{max}$ | 0.03 | 0.20 | 6.00 |

Km : mM
Vmax : nmol/mg protein/min
HW : Hanes-Wolf plot
LB : Lineweaver-Burk plot
2A3MPNO : 2-amino-3-methylpyridine-N-oxide
2A3HMP : 2-amino-3-hydroxymethylpyridine
2A3M5HP : 2-amino-3-methyl-5-hydroxypyridine

idine; no evidence was found to suggest that metabolic attack occurred at the *exo*-amino group. Previously Sugimura *et al.* /1/ had shown that only those metaoblites of aniline produced through oxidation of the amino group retained the comutagenic activity shown by the parent aniline. As comutagenesis tests are usually conducted using a rat hepatic S9 preparation derived from animals pretreated with arochlor 1254, we thought that our previous results were explicable in that any *exo*-N-oxidised metabolite would be produced at only low levels by hepatic tissues from untreated animals; hence the present experiments utilising S9 preparation from arochlor 1254 pretreated rats. Contrary to our expectations no 2-hydroxylamino-3-methylpyridine, 2-nitroso-3-methylpyridine or 2-nitro-3-methylpyridine could be detected even under the experimental conditions (Figure 1) in which the optimal formation of metabolites had been established.

As shown in Figure 4, the effect of variation of substrate concentration on the *in vitro* metabolism of 2A3MP, 2 μmol substrate per incubate could be used for formation of 2A3MPNO, 2A3HMP and 2A3M5HP. When either $S/V_o$ (S=mM substrate concentration and $V_o$ = nmol product per mg protein per min) was plotted against S (Hanes-Wolf plot) or $1/V_o$ was plotted against $1/S_o$ (Lineweaver-Burk plot), a linear relationship was obtained The $K_m$ and $V_{max}$ values for the C- and 1-N-oxidation of 2A3MP are summarised in Table 2. These results show that the $K_m$ value for N-oxidation of 2A3MP (2A3MPNO), i.e. 1.53 mM and 1.73 mM respectively, are similar using the Hanes-Wolf plot and the Lineweaver-Burk plot. In the case of 2A3HMP the $K_m$ value using the Hanes-Wolf plot was lower (1.01 mM) and using the Lineweaver-Burk plot was higher (1.83 mM) than 2A3MPNO. Comparing 1-N-oxidation and methyl hydroxylation it can be seen they were only slightly different using the two methods of determination, whereas in the case of 5-hydroxylation of 2A3MP the results were similar from both methods (3.06 mM and 3.44 mM) but distinctly different from those of the former metabolites. These differences in the $K_m$ values suggest that different isozymes of cytochrome P450 are responsible for the 5-hydroxylation pathway whereas both N- and methyl oxidation of 2A3MP are carried out by enzymes having similar catalytic characteristics.

The present results contribute little to our understanding of the process of comutagenicity of 2A3MP. It was previously thought that N-hydroxylation of the *exo*-amino group may be a prerequisite for comutagenic activity as occurs for aniline /1/; however no metabolite

derived via N-hydroxylation of the 2-amino group was detected even when the incubation conditions were optimised and using a hepatic S9 preparation derived from arochlor 1254 pretreated rats as enzyme source. It may be that any N-hydroxy metabolite is only present at concentrations below that detected by the present HPLC system due to low rates of production or higher rates of retroreduction /5/. Alternatively the N-hydroxy compound may be so reactive that it binds covalently with cellular constituents and is lost as insoluble bound material and not detected. Norharman may act as an activator of the metabolic N-hydroxylation of 2A3MP allowing this process to occur only in its presence.

The enzymology of ring and methyl hydroxylation, N-oxidation of 2A3MP and the effect of norharman on metabolic pathways will be the subjects of later communications.

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