Cellular and Subcellular Studies of the Biotransformation of Hexamethylmelamine in Rat Isolated Hepatocytes and Intestinal Epithelial Cells

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ABSTRACT

The antitumor agent hexamethylmelamine is subject to oxidative metabolic conversion in rat isolated liver and small intestinal cells (conversion 40 times higher in hepatocytes). This N-demethylation is mediated by cytochrome P-450 in the microsomal fractions, and in mitochondrial preparations it has been found to occur via N-methylpentamethylmelamine. Somehow, pentamethylmelamine, hydroxymethylpentamethylmelamine, or an intermediary metabolite becomes trapped in the intact cell, but the nature of the adduct formed is still unresolved. Pretreatment of rats with 3-methylcholanthrene p.o. caused a 5-fold increase in hexamethylmelamine turnover. Phorone administered in vivo prior to cell preparation (liver and gut) caused an increase in pentamethylmelamine production. The latter results together with results of adding glutathione to cell incubations demonstrate that glutathione contributes to the regulation of cytochrome P-450-mediated N-demethylation of hexamethylmelamine.

INTRODUCTION

HMM2 (Chart 1) is a synthetic S-triazine derivative, which has demonstrated activity against a wide variety of solid human tumors (7, 22). There is evidence that metabolism of HMM is required for antitumor activity (30), but the mechanism involved is not known. HMM is extensively metabolized to demethylated metabolites in vivo and in vitro experiments (3, 12, 21, 34) and in vitro experiments (3, 35) in which hepatic (microsomal) preparations are practically always used. Since neither HMM nor its demethylated products have alkylating activity (4, 34), other species must be responsible for the observed covalent binding in vitro and in vivo (5, 15).

HMPMM (Chart 1) has been found to be produced in vitro in liver microsomal incubations (16) and to be significantly more cytotoxic to tumor cell lines than is HMM itself (13, 30). It has also been demonstrated (5) that [ring-14C]HMPMM binds covalently to microsomal protein (in the absence of NADPH-regenerating system) and, to a much greater extent, to calf thymus DNA. The general view is that HMPMM is formed during the hepatic N-demethylation of HMM as an intermediate and is involved in the covalent binding to tissue macromolecules. HMPMM could be subject to conjugation, as was shown for a carbinolamine intermediate in the demethylation of N,N-dimethyl-4-aminobenzene (11). The link with the antitumor activity is not known; furthermore, HMPMM has never been conclusively demonstrated in plasma.

Recent experiments in our department have shown that HMM is subject to extensive intestinal wall metabolism in vivo (21) and have revealed first-pass effects in rat liver and in the intestinal wall of 73 and 71%, respectively. This presystemic metabolism could mean either an extrahepatic activation different from hepatic biotransformation or simply an extra contribution to higher plasma levels of HMPMM. Previously, we reported (8) that mitochondrial preparations of intestinal cells were able to produce HMPMM. We now report further studies on HMM metabolism in vitro and compare results obtained with isolated hepatocytes and intestinal cells. We also studied the metabolic conversion of HMM at subcellular level (microsomes, 9000 g supernatant, mitochondria).

MATERIALS AND METHODS

Chemicals

HMM was obtained from Ofichem (Gieten, The Netherlands). HMM hydrochloride was prepared as described previously (21, 33). The [ring-14C]HMM (6.8 mCi/mmol) was supplied by Dr. Robert Engle, Chemical Resources Section, Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute. The N-demethylated metabolites of HMM were kindly provided by Dr. C. J. Rutty (Institute of Cancer Research, Sutton, Surrey, United Kingdom). HMPMM was kindly provided by Dr. A. Gescher and Dr. S. P. Langdon (Aston University, Birmingham, United Kingdom). Glucose-6-phosphate dehydrogenase (Grade I), NADP+, glucose 6-phosphate, ADP, and ATP were purchased from Boehringer Mannheim (Mannheim, West Germany). 3-Mc and phorone (95% purity) were obtained from Fluka AG and Aldrich (Beaune, Belgium), respectively. All other solvents and reagents were of analytical (p.a.) grade. SKF-525A was a gift from Smith Kline & French. Ochotamine, GSH, and 2-vinylpyridine were obtained from Merck AG (Darmstadt, West Germany). GSSG reductase (EC 1.6.4.2; type III; No. G-4751) and GSSG were purchased from Sigma.

Animals and Pretreatment

Adult male Wistar rats (Cpb:WU) (230 to 250 g) were obtained from TNO (Zeist, The Netherlands). In order to study the effect of 3-MC or phorone on intestinal metabolism, we administered 3-MC (20 mg/kg) or phorone (200 mg/kg) to the animals, in 1.0 ml corn oil by stomach tube, 24 hr before preparing the intestinal mucosal cells. To obtain hepatic...
GSH depletion, phorone (200 mg/kg) was injected i.p. 2 hr before the isolated hepatocytes were prepared. Before and after pretreatment, rats were allowed free access to tap water and a commercially available diet (Muracor-1; Trouw, Putten, The Netherlands).

Isolation of Cells and Preparation of Subcellular Fractions

Isolation of Cells. The animals were killed (between 9 and 10 a.m.) by cervical dislocation, and the small intestine was immediately flushed free of its contents with ice-cold phosphate-buffered saline (90 mM NaCl, 9 mM KH2PO4, and 34 mM Na2HPO4•2H2O, pH 7.4). All further work on isolating intestinal cells was done at 0–4°C with materials made of polypropylene. A high-frequency low-amplitude vibration method was used to isolate intestinal epithelial cells. Details have been described previously (9). Isolated mitochondria were prepared in basically the same way as described by Lernhoff et al. (19). Isolated cells obtained from 0.125 g intestine [7.5 x 10^6 cells, 3.0 ± 0.1 mg protein] were incubated in a final volume of 3.0 ml carbogen-saturated KRB.

Preparation of Subcellular Fractions. Microsomes were prepared from cell suspensions by homogenization in 50 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA using an Ultra-Turrax (Janke & Kunkel KG, Staufen in Breisgau, Switzerland) (11,000 rpm, 3 bursts of 10 sec); and the homogenate was used to obtain microsomal preparations, as described previously (9). Isolated mitochondria were prepared in basically the same way as described by Lemnhoff et al. (19). Isolated cells were suspended in phosphate buffer; EDTA containing 100 mM KCl, 5 mM MgCl2, and 1 mM ATP and homogenized (see above). The homogenate was centrifuged for 5 min at 1000 x g. The supernatant was centrifuged again for 5 min at 1,000 x g and subsequently for 10 min at 12,000 x g. The pellet (a rough mitochondrial preparation) was resuspended in ice-cold buffer and was used within 1 hr of preparation.

Incubation of Cells and Subcellular Fractions

Isolated cells obtained from 0.125 g intestine [7.5 x 10^6 cells, 3.0 ± 0.2 (S.E.) mg protein] or 0.005 g hepatic tissue [2.5 x 10^6 cells, 1.0 ± 0.1 mg protein] were incubated in a final volume of 3.0 ml carbogen-saturated KRB. Cells were incubated within 2 hr of isolation in polypropylene incubation vessels in a shaking water bath (60 cycles/min) at 37°C. Microsomes obtained from 0.5 g intestine (1.0 ± 0.2 mg protein) or 0.05 g hepatic tissue (0.9 ± 0.1 mg protein) and mitochondria from 0.125 g intestine or 0.005 g liver were incubated in a final volume of 3.0 ml 50 mM phosphate buffer (pH 7.4) containing EDTA (0.1 mM), NADP+ (0.5 mM), glucose 6-phosphate (4.2 mM), MgCl2 (4.2 mM), and glucose-6-phosphate dehydrogenase (0.3 IU/ml).

After a 10-min preincubation, the reaction was started by the addition of 0.5 ml HMM hydrochloride solution in water (final concentration between 1 and 200 μM). The reaction was stopped at appropriate times between 0 and 60 min by the addition of 375 μl TCA (15%), and the mixture was centrifuged for 5 min at 1000 x g.

Inhibitors were added during preincubation in 30 μl water (SKF-525A, GSH, metyrapone, KCN) or in 10 μl methanol (octylamine); control incubations received only solvent. Inhibitors and/or solvents had no effect on cell viability during 30 min incubation at 37°C. Cells were treated in vitro with phorone (1 to 5 mM) by adding 50 μl of a methanolic solution of phorone to 10 ml of cell suspension (5 x 10^5 hepatocytes/ml or 15 x 10^5 cells/ml for intestinal cells) in KRB. Viability of cells incubated for 60 min with phorone was not different from that in cells preincubated with methanol only. However, in both cases, viability was found to decrease from about 90% immediately after isolation to 50 to 55% after 60 min preincubation.

HPLC Analysis of HMM and Metabolites

The conversion of HMM into PMM was determined using HPLC and UV detection (λ = 228 nm). The possible presence of other metabolites (N2,N2,N4,N4-tetramethylmelamine, trimethylmelamine) as products of further demethylation was checked with a GC assay, which has been described previously (17). The supernatants of TCA-treated and centrifuged samples (above) were neutralized with 0.5 N NaOH, and 20 to 50 μl were injected into the liquid chromatograph after the neutralized supernatants had been allowed to stand overnight.

HPLC was performed using a 6000–A solvent delivery system and the WISP 710B automatic injection system from Waters Associates. The chromatographic column (30 x 0.46; Lichrosorb 10 C18, Chrompack) was used at a head pressure of 3000 psi and at a flow rate of 1.5 ml/min. The mobile phase consisted of methanol:phosphate buffer (0.01 M, pH 7.4, with 0.01 M EDTA), 60:40 (w/w). Eluted compounds were monitored at 228 nm (range, 0.04) using a Pye Unicam LC-UV variable-wavelength detector. Peak heights were used to quantify the amount of HMM (retention time, 8.6 min) and PMM (Rf, 5.4 min) in the samples (Chart 2). The assay has a detection limit of 0.5 ng PMM (S/N = 3) and a high reproducibility (S.D. <2%, 2 to 30 ng injected). HMPMM was detected at a retention time of 4.8 min. HMPMM was identified using gas-liquid chromatography-MS and HPLC, as described previously (8). In order to identify HMPMM with gas-liquid chromatography-MS, incubations were stopped with 2.0 ml of ethyl acetate, 50 μl of 0.5 N NaOH were added, the mixture was vortexed for 1 min, and 1.0 ml of the organic layer was reduced to dryness at 40°C. HMPMM was identified as a metabolite after derivatization with 20 μl of bistrimethylsilyl trifluoroacetamide. Mass spectra were recorded on a Kratos MS80 mass spectrometer equipped with a Carlo Erba 4160 gas chromatograph (MS: electron energy, 70 eV; ionizing current 100 μA; source temperature, 250°C). The used GC column (200 cm x 2 mm inside diameter) was slanized and packed with 3% OV-17. The operating conditions were: injection port temperature, 260°C; carrier gas (helium) flow rate, 15 ml/min. The oven temperature was programmed following each injection: 2 min isothermal at 180°C; then 4°/min from 180°C to 260°C. HMPMM is detected at a retention time of 14.23 min and was characterized by comparison of the mass spectrum with standard compound chromatogram. The ratio of the M-283 (silylated HMPMM minus CH3) and M-298 (silylated HMPMM minus CH2) peaks was 1.00 and was used to distinguish HMPMM in mitochondrial incubations from an endogenous peak (Rf 13.40).

Other Biochemical Techniques

Cell viability was assessed by lactate dehydrogenase leakage (10) in the case of intestinal epithelial cells. Trypan blue dye exclusion and lactate dehydrogenase leakage were used for hepatocytes, showing results similar to those reported previously (6). The amount of cytochrome P-450 was estimated by means of a dithionite difference spectrum (14) (ε = 100 1000 M⁻¹ cm⁻¹) after CO reduction in microsomes and by subtracting the succinate CO-P-450 value from the dithionite-P-450 value.
to eliminate interference from other cytochromes in mitochondrial fractions [20].

We checked the functional state of isolated mitochondria by studying the respiratory control, using succinate as the oxidizable substrate. Respiratory rates were measured with a Clark-type oxygen electrode (YSI type LN 9981) in 0.75 ml of a medium consisting of 30 mM KCl, 10 mM MgCl₂, 5 mM EDTA, 10 mM Tris-Cl and 10 mM K₂HPO₄ (pH 7.5). Respiration was initiated by the addition of succinate (10 mM final concentration), and phosphorylation was initiated by the addition of 0.4 μmol of ADP.

Total glutathione and GSSG were determined using a kinetic assay in which catalytic amounts of GSSG and GSH and GSSG reductase bring about the continuous reduction of 5,5'-dithiobis(2-nitrobenzoic acid) by NADPH [1]. The formation of 3-thio,5-nitrobenzoic acid was followed spectrophotometrically at 412 nm in samples pretreated with 2-vinylpyridine (binds GSH) and in untreated samples. The difference in GSH equivalents revealed the GSH content. 2-Vinylpyridine was freshly dissolved in ethanol.

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RESULTS

When studying HMM disposition in whole cells isolated from the small intestine and/or liver of control rats, we observed a discrepancy between the amount of HMM converted and the amount of PMM produced (Chart 2, A and B; Table 1). Conditions of incubation ensured that no further demethylation of PMM occurred (checked with the GC assay) and that nonspecific binding to protein or mucus of HMM was less than 2%.

PMM production was almost completely (>95%) inhibited when SKF-525A (1 mM) or octylamine (1 mM) was coincubated with HMM in cell suspensions of both liver and small intestine. However, in these same incubations, HMM conversion was only partly inhibited (Chart 2, C and D). When subcellular fractions of small intestinal cells were used, we showed previously (8) that HMM conversion equalled PMM production (see also Table 1). This also holds for microsomes and 9000 x g supernatants prepared from isolated hepatocytes, but not for the mitochondrial fraction (Table 1). Hepatic mitochondrial turnover resembled that in whole cells in that HMM turnover was greater than PMM production and was only partly inhibited (50%) by the addition of SKF-525A (1 mM). Omission of the NADPH-regenerating system (Chart 3) did not reduce hepatic mitochondrial HMM turnover to zero, whereas HMM turnover was shown previously (8) to be diminished by the same omission. A combined GSH 100,000 x g supernatant addition again inhibited mitochondrial PMM production, as was observed previously using intestinal subcellular fractions (8) (Chart 3). After the HMM turnover rates had been corrected for cell viability, recoveries, and contamination (Table 1), the summation of HMM turnover rates (at 20 μM) of mitochondria and microsomes was found to conform to the cellular turnover of HMM. This held for both cell types. However, the same calculation yielded a PMM production different from that observed in whole cells. Perhaps an unknown metabolite of

![Chart 2. HMM conversion (O, D, C) and corresponding PMM formation (□) in intestinal cells (A, C) and hepatocytes (B, D). Isolated cells were incubated with 20 μM HMM as described in the text, stopped at various time intervals with TCA, and analyzed by HPLC. A and B show HMM conversion (O) and PMM production (□) in control incubates; C and D show HMM conversion in analogous incubations containing 1 mM SKF-525A (C) or 1 mM octylamine (D) as compared to controls (□). No PMM was detected when inhibitors were present. Results are the means of triplicate incubations with 3 different batches (n = 9). Bars, S.E. If no bars are indicated, S.E. is smaller than the symbol.](chart2.png)

Table 1

<table>
<thead>
<tr>
<th>In vitro preparation</th>
<th>Small intestine</th>
<th>Liver</th>
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<tr>
<td></td>
<td>HMM conversion</td>
<td>PMM production</td>
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<tr>
<td>Microsomes</td>
<td>1.00 ± 0.10</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td>9000 x g supernatant</td>
<td>0.50 ± 0.09</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.29 ± 0.10</td>
<td>1.15 ± 0.10</td>
</tr>
<tr>
<td>Cells</td>
<td>2.37 ± 0.53</td>
<td>0.77 ± 0.06</td>
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* Corrected for cell viability (90%), mitochondrial (75%; see Ref. 8) and microsomal recovery (45%; see Ref. 10), and microsomal contamination in mitochondria (25%; see Ref. 8).

** Mean ± S.D. of the indicated number of cell batches (n).

* Significantly different from HMM conversion at p < 0.001, Student’s t test paired samples.

* Significantly different from HMM conversion at p < 0.01, Student’s t test paired samples.
HMM or PMM becomes trapped in the cell as a conjugate or becomes bound (covalently) to protein.

Several experiments were devised to test the above hypotheses: (a) Incubations of intestinal cells with HMM \( (20 \mu M) \) were stopped with TCA and treated with \( \beta \)-glucuronidase: arylsulfatase or hydrochloric acid as depicted in Chart 4. However, no increase in PMM or HMM concentration was observed. One could imagine that hydrolysis of possible glucuronic acid or GSH conjugates could have led to such an increase. (b) Incubation of intestinal cells with \([\text{ring}^{-14}\text{C}]\)HMM \( (20 \mu M) \) revealed that only 2% of total radioactivity was recovered from the acid precipitate after 30 min of incubation. Radioactivity, however, was easily removed by an extraction procedure, as described previously by Garattini et al. (15), which indicates that it was not bound covalently. (c) More indirectly, we studied the effects of phorone and 3-MC administered \( \text{in vivo} \) in corn oil. The effects of 3-MC, phorone, and corn oil on HMM turnover and PMM production are listed in Table 2 for intestinal cells and in Table 3 (phorone only) for isolated hepatocytes. The results for intestinal cells stem from experiments in which the animals were given a single p.o. dose of corn oil or the drug in corn oil 24 hr prior to cell preparation. Pretreatment of rats with phorone p.o. 2 hr prior to cell preparation (normal procedure for hepatic GSH depletion, i.p.) or \( \text{in vitro} \) incubation (60 min, 37°) of intestinal cell suspensions with up to 5 \( \mu M \) phorone did not result in a depletion of glutathione, nor did it increase PMM production (data not shown). In hepatocytes, only the phorone effect was studied, the animals being given phorone i.p. 2 hr before liver cannulation. This time-dose combination was previously shown to deplete nearly 95% of hepatic GSH (23). As can be seen in Table 2, 24 hr after the p.o. administration of phorone, there was a marked increase in PMM production. However, we could not detect a depletion of GSH in the intestinal cells of animals that had been treated with phorone. A more pronounced increase in PMM formation was observed in hepatocytes from rats treated i.p. with phorone, but in these cells we did observe an 80% depletion of GSH. Again the turnover of HMM was not affected. When GSH or GSSG (3 \( \mu M \)) was added to cell suspensions of control and phorone-pretreated rats, there was a significant decrease in the (increased) PMM production in cells of phorone-treated animals only. HMM turnover remained unaffected.

A single dose of 3-MC, administered 24 hr prior to the preparation of intestinal cells, resulted in a 5-fold increase in the HMM turnover rate and a 12-fold stimulation of PMM production. As a result, PMM constituted a relatively larger fraction (63%) of HMM turnover in these cells than in cells from control rats (23%).

**Chart 4.** HMM conversion (○) and PMM production (●) as measured in intestinal cell incubations with HMM \( (20 \mu M) \). A, cell incubations stopped with TCA, supernatant analyzed by HPLC; B, supernatant \( (0.5 \text{ ml}) \) treated with 0.5 ml \( (1000 \text{ FU}) \) \( \beta \)-glucuronidase:aryl sulfatase in 0.1 M acetate buffer, pH 5.0, after a 48-hr incubation period at 37° analyzed by HPLC; C, 0.5 ml supernatant was mixed with 0.5 ml 1 M HCl, heated for 60 min at 100°, and subsequently analyzed by HPLC. Bars, S.E. of hepatic GSH (23).

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Influence of p.o. pretreatment with phorone or 3-MC on HMM turnover and PMM production in isolated small intestinal mucosal cells

Animals received phorone (200 mg/kg) or 3-MC (20 mg/kg) in 4.0 ml corn oil per kg body weight, controls receiving corn oil only, by stomach tube. Twenty-four hr after injection, rats were killed, and intestinal cells were prepared. Pretreatments did not affect cell viability (85 to 90%). All data are corrected for a cell viability of 90%.

Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Controls, oil</th>
<th>Phorone</th>
<th>3-MC</th>
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<tr>
<td>No addition</td>
<td>66.8 ± 6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.0 ± 6.3</td>
<td></td>
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<tr>
<td>HMM</td>
<td>9.0 ± 2.1</td>
<td>61.0 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>SKF-525A (1 mw)</td>
<td>33.7 ± 6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Octylamine (1 mw)</td>
<td>54.7 ± 6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH/GSSG (3 mw)</td>
<td>74.5 ± 4.2</td>
<td>53.0 ± 4.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>HMM</td>
<td>10.3 ± 1.0</td>
<td>49.3 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> Paired data are presented for HMM turnover and PMM production. Mean ± S.E. of 2 batches. Significantly different from same parameter in control rats (p < 0.01, Student's t test). ND, not detectable.

However, the amount of PMM produced was still significantly lower than the amount of HMM converted (p < 0.01, Student's t test).

DISCUSSION

We have shown that HMM is metabolized in a similar way by isolated intestinal cells and hepatocytes. Absolute turnover rates, however, are about 40 times higher in hepatocytes when expressed per g organ (or per mg protein). The results obtained with SKF-525A, octylamine, and 3-MC suggest that cytochrome P-450 monooxygenases are involved. The metabolic capacity for HMM turnover in intestinal cells (V<sub>max</sub> 5 nmol/min/g) is considerably higher than for the cytochrome P-450-mediated O-deethylations of phenacetin and 7-ethoxycoumarin (V<sub>max</sub> <0.3 nmol/min/g) (9).

From the results that we obtained for microsomes and mitochondria isolated from small intestinal cells, we suggested (8) a mechanism that might be responsible for N-demethylation of HMM, catalyzed by cytochrome P-450 (see Chart 5). In summary, we suppose that HMPMM is formed as a relatively stable intermediate in both organelles. In mitochondria, however, the environment (lipid composition, membranes) where HMPMM is formed is quite unique, being inaccessible to many compounds. HMPMM formed in the microsomal system is immediately converted into PMM (and formaldehyde) by an (unknown) enzymatic factor, but this enzymatic factor is not present in mitochondria.

The results that we obtained with hepatic subcellular preparations seem to confirm this hypothesis. Furthermore, when HMPMM is dissolved in liver 100,000 x g supernatant, it is degraded enzymically to PMM within minutes (after denaturation of the supernatant, decomposition reverts to the spontaneous reaction, t<sub>1/2</sub> = 1.75 hr); but how are we to explain the results obtained with cell incubations where the HMM conversion was always greater than the spontaneous reaction (lipid composition, membranes) where HMPMM is formed is quite unique, being inaccessible to many compounds. HMPMM formed in the microsomal system is immediately converted into PMM (and formaldehyde) by an (unknown) enzymatic factor, but this enzymatic factor is not present in mitochondria.

A summation of HMM turnover rates of mitochondria and microsomes (Table 1) is equivalent to the cellular turnover of HMM. This means that we can explain the turnover of HMM, but somehow a metabolite (HMPMM or a PMM metabolite) must have become trapped in the intact cell, resulting in an apparently lower PMM production. To explain the effect(s) of SKF-525A on cellular biotransformation, we suggest either that the inhibitor is not bioactivated (a prerequisite for its action) in mitochondria or that it cannot pass through the mitochondrial membranes which are lying structurally intact in the cell. As a result, only N-

| Chart 5. Schematic representation of the microsomal and mitochondrial N-demethylation of HMM leading to PMM and formaldehyde or (GSH-) adducts of HMPMM or the thioether species. Segmented arrows indicate where 3-MC and phorone are thought to influence HMM biotransformation.
demethylation in the endoplasmatic reticulum (being responsible for almost all PMM formed; see above hypothesis) is inhibited.

Worzalla et al. (35) studied the effect of microsomal enzyme inducers on HMM N-demethylation activity in vivo (\(^{14}C\)O\(_2\) production) and in vitro (formaldehyde) in hepatic microsomes. When they used phenobarbitone, 3-MC, prochlorperazine, and chlorpromazine, they found that only the first-mentioned compound caused a significant induction of demethylation activity. Covalent binding in rat hepatic microsomes (5) and to calf thymus DNA is also increased by previous Phenobarbitone treatment of the laboratory animals. When we used intestinal cells as an in vitro system, we observed a 5-fold increase in HMM turnover after pretreating rats with 3-MC, known as a P-448-type inducer (25).

How can we fit our results of 3-MC induction into the previous hypothesis? It was shown previously (20) in intestinal epithelial cells of rats pretreated with 3-MC (p.o.) that only microsomal cytochrome P-450 and not the mitochondrial one is induced. According to our theory, all the HMPMM formed in microsomes is converted to PMM. Therefore, when microsomal N-demethylation is increased, relatively more PMM has to be produced, as indeed was observed.

The increase of PMM formation in intestinal cells and/or hepatocytes after in vivo phorone administration can be attributed to reduced conjugation of HMPMM or the iminium ion (originating from HMPMM (Chart 5)) with GSH. Depletion of GSH in isolated hepatocytes correlates well with the observed increase in PMM formation. It is strange, however, that we could not detect lowered GSH levels in intestinal cell homogenates. The specificity of our method of measuring GSSG/GSH is governed by that of GSSG reductase and the assumption that 5,5'-dithiobis(2-nitrobenzoic acid) leads to the quantitative formation of GSSG without interference of other kinds. It has been demonstrated (32) that a 100-fold excess of cysteine does not influence the assay significantly. Therefore, it is not likely that our assay is affected by other thiols present in intestinal cells.

Coles et al. (11) isolated a GSH adduct apparently derived from a reactive carbinolamine intermediate formed during the demethylation of a metabolite of diaminoazobenzene. If a glutathione conjugate is formed from HMPMM or the iminium ion, then acid hydrolysis of incubations (Chart 4) should have caused an increase in PMM. Such an increase was observed by Colombo et al. (12) in urine samples of rats dosed i.v. with HMM and PMM. HMPMM appeared to react with a large excess of GSH in buffered solution to yield a product which upon thin-layer chromatographic analysis turned pink when sprayed with ninhydrin; this product, presumably glutathionymethylene-PMM, decomposed upon subsequent isolation attempts. However, this reaction product was not a biliary metabolite in bile-cannulated rats and was not present in the plasma of mice, both species having been given HMPMM. No compound with the chromatographic properties of the adduct could be found in incubations of HMM with mouse hepatocytes either.3

It is possible that phorone acts at a level different from that of GSH depletion. Several investigators have suggested that an iminium ion is formed as an intermediate in the N-demethylation of nicotine (26), N-methylcarbazole (31), diaminoazobenzene (11), and HMM (5). This iminium ion can be converted to the carbinolamine both by peroxide and by NADPH-supported cytochrome P-450 oxidations (31). In addition to acting via GSH depletion, compounds like phorone, diethyl maleate, and cyclohexenone are known to interact with the microsomal monooxygenase system (28), resulting in type I binding spectra. From the latter study, phorone seemed the best agent for achieving GSH depletion, since it has the highest apparent binding constant. However, this interaction is reported (28) to cause lipid peroxidation directly. If the balance between the oxidase and the oxygenase activity of cytochrome P-450 is disturbed by the pretreatment of rats with phorone (enhanced lipid peroxidation), one would expect HMM metabolism to alter. HMM turnover in subcellular preparations of intestinal cells is not affected after pretreating rats with phorone (data not shown). The latter observation indicates that the intact cell is a prerequisite for finding enhanced PMM formation.

An attempt to mimic the (hypothetical) lipid peroxidation by the addition of cumene hydroperoxide to subcellular incubations showed that peroxide-mediated N-demethylation was very small as compared to NADPH-mediated demethylation (Chart 3). However, this evidence is certainly not conclusive, because the peroxide was added exogenously and in a different form.

Conversion of HMM in intestinal cells helps to clarify its considerable intestinal first pass (21). It is not clear what causes the considerable mitochondrial turnover in both intestine and liver. Previously (8), we suggested that mitochondrial cytochrome P-450 was responsible for the formation of HMPMM in intestinal mitochondrial preparation (after checking a possible function for the monoamine oxidase). Now we report that liver mitochondria (containing much less cytochrome P-450) also convert HMM.

We still do not know how precisely the intestinal conversion of HMM [now observed in vitro and in vivo (21)] contributes to the antineoplastic activity of HMM. It is certain that this extrahepatic conversion could result in higher plasma levels of HMPMM. However, thus far it has not been possible to demonstrate any HMPMM in vivo. Perhaps there is a difference between hepatic and intestinal conversion, which could imply an extrahepatic activation step. In the present study, however, we observed no striking differences between the conversion of HMM in either organ. In subsequent studies, we are concerned with the elucidation of the conjugate formed and with the investigation of the role of other enzymes involved.

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