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STUDIES OF THE MODE OF ACTION OF ANTITUMOUR TRIAZENES AND TRIAZINES—III. METABOLISM STUDIES ON HEXAMETHYLMELAMINE*

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Abstract—There is good evidence that the antitumour agent hexamethylmelamine (HMM) undergoes oxidative metabolic activation which might occur in the liver and/or extrahepatically. The hepatic microsomal *N*-methylmelamine metabolizing enzymes were investigated in mice and exhibited different affinities for different melamine derivatives. The apparent K_m values are 0.09 mM for HMM, 0.23 mM for pentamethylmelamine, 0.91 mM for 2,2,4,6-tetramethylmelamine and 1.7 mM for trimethylmelamine. HMM inhibited its own metabolism *in vitro* at substrate concentrations >0.5 mM. Its hepatic microsomal *N*-demethylation rate was reduced when the mice were pretreated with the hepatic glutathione depleting agent methyliodide. Injection of hexaethylmelamine, a derivative of HMM without antineoplastic properties against the M5076 sarcoma in mice, lead to plasma concentrations of drug and metabolite pentaethylmelamine which were only a fraction of the drug and metabolite levels achieved after a similar dose of HMM.

Hexamethylmelamine (HMM) (Fig. 1) is an antitumour drug used with moderate success in the treatment of ovarian and lung cancer [1]. Its mode of action is not well understood. There is however good evidence that HMM requires metabolic activation. Whereas the drug itself is cytotoxic *in vitro* only after prolonged exposure to cells [2–4] one of its major *in vitro* metabolites *N*-hydroxymethylpentamethylmelamine(*N*-hydroxymethyl-PMM) [5] (Fig. 1) is highly cytotoxic when incubated with a series of murine [2] and human [3, 4] tumour cell lines. Furthermore studies using mouse lymphoma cells *in vitro* have shown that HMM, itself quite non-toxic in this system, is biotransformed by rat liver microsomes to directly cytotoxic products [6]. The latter study also demonstrated the requirement of *N*-methyl moeities in the melamine molecule for activity against the PC6A plasma cell tumour in mice. Hexaethylmelamine (HEM) is not an antitumour agent in this system and the major *N*-demethylated *in vivo* metabolites of HMM, 2,2,4,6-tetramethylmelamine (tetraMM), 2,4,6-trimethylmelamine (triMM), 2,4-dimethylmelamine and methylmelamine (Fig. 1) possess only marginal activity or are devoid of antitumour activity. It therefore appears that the metabolic C-hydroxylation of HMM to *N*-hydroxymethyl-PMM (Fig. 1) is an activation reaction whereas the formation of the major *N*-demethylated metabolites is a detoxification reaction. Oxidative *N*-demethylation is the sole metabolic pathway which HMM is known to undergo. Generally, this ubiquitous pathway in xenobiochemistry [7] leads to pharmacologically inert metabolites.

In this study the attempt was made to characterize the hepatic enzymes which catalyse the metabolism of HMM and its *N*-desmethyl metabolites. This was performed by kinetic analysis of the reaction catalysed by mouse microsomal enzymes *in vitro* using as substrates HMM, pentamethylmelamine (PMM), tetraMM, triMM and for comparative purposes aminopyrine (AP), a 'model' substrate for which *N*-demethylation *in vitro* [8] and *in vivo* [9] has been well described. The *N*-demethylation of the HMM metabolites was studied in order to verify the hypothesis that the long plasma elimination half lives of the HMM metabolites tetraMM and triMM as compared to those of HMM and PMM [10, 11] reflect differences in the affinities of the differently methylated melamines for the mixed function oxygenases.

To characterise the HMM metabolizing enzymes further we investigated the influence of hepatic levels of the non-protein thiol glutathione (GSH) on HMM metabolism. This study was done in analogy to an investigation in which the metabolic *N*-demethylation of *N*-dimethylaminoazobenzene, a liver carcinogen, was found to be controlled by hepatic GSH levels [12]. In order to ascertain that the peculiar structural requirement of *N*-methyl moieties for the antitumour activity of HMM [6] observed in a murine tumour model is also applicable to other mouse tumours sensitive to HMM we tested HEM and melamine (Fig. 1) for activity against the murine

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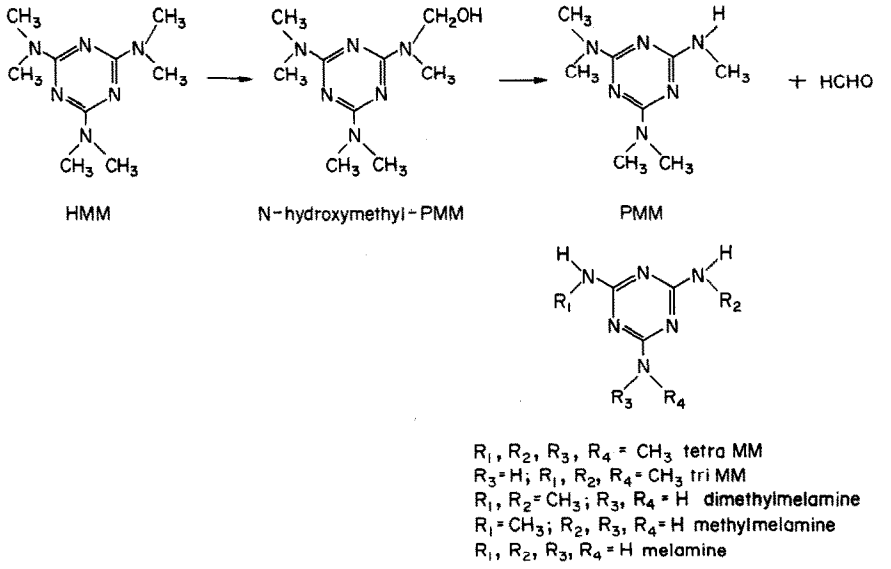

Fig. 1. Metabolites of HMM.

M5076 reticulum cell sarcoma.* Finally, in order to establish if there is a pharmacokinetic basis for the structural requirement of *N*-methyl moieties in the melamine molecules for antitumour activity we studied the plasma disposition of HEM, and compared it with the plasma profile reported for HMM in mice [10].

MATERIALS AND METHODS

Compounds and animals. HMM and its metabolites were synthesized in our laboratories by Professor Stevens and Dr. Simmonds according to published methods [14]. All other chemicals were commercially available.

In the antitumour tests and the *in vivo* metabolism experiments female C57 Black/6J or BDF_1 mice (20–25 g) were used. For the *in vitro* experiments livers were obtained from male Balb C mice [20–25 g). Drugs dissolved in acetone/arachis oil 1:5 were administered i.p.

Antitumour assay. A suspension of 10^6 M5076 tumour cells from a routine passage grown as a solid i.m. tumour in mice was injected i.m. into the left hind leg of groups of ten mice. Drugs were administered daily for up to 17 days, i.e. approximately half the life span of the tumour bearing animals. Mean tumour volumes were measured by calipers every fourth day from day 12 until death and the mean tumour volume index (TVI) calculated by the standard method [15].

Metabolic incubations. 9000 *g* liver fractions were prepared by differential centrifugation of a 10% homogenate in 0.25 M sucrose. Microsomes were prepared in the usual way [16] and suspended in Earl's buffer so that 200 mg equivalent of the net weight of the liver was suspended in 0.5 ml buffer. Substrate concentrations ranged from 0.02 to 5 mM. Substrates were dissolved in acetone or dimethylsulphoxide, of which a volume of 0.1 ml was added to the mixtures. The incubation mixtures in Earl's buffer were fortified with cofactors which generated 1 mM NADPH in a final volume of 2.5 ml. All incubations were performed in duplicate. After 20 min incubation shaking under air the mixtures were either deproteinised with 0.5 ml of a 20% trichloracetic acid solution to prepare the samples for the colorimetric determination of formaldehyde or treated with 0.5 ml of 1 mM NaOH to prepare the samples for the g.l.c. assay of the alkylmelamines. Microsomal protein was determined according to Lowry *et al.* [17].

Analytical methods. Products of oxidative *N*-demethylation, formaldehyde or formaldehyde precursors [18], were measured colorimetrically by the method of Nash [19] as described by Werringloer [20]. Absorbances were read in a Beckman Acta TM5 spectrophotometer. The *N*-alkylmelamines and their dealkylated metabolites were quantified in samples of the incubation mixtures and of plasma by a gas chromatographic assay according to D'Incalci *et al.* [21] using a Pye 204 chromatograph fitted with a FID detector. Ether was used to extract *N*-alkylmelamines from the incubation mixtures or plasma samples. The recoveries for the respective agents were HMM $90.8 \pm 4.7\%$ ($n = 9$), PMM $89.4 \pm 7.5\%$ ($n = 19$), tetraMM $89.5 \pm 6.0\%$ ($n = 10$) HEM $76.6 \pm 12.5\%$ ($n = 5$).

Determination of the enzyme kinetic constants. Rates of formaldehyde production or substrate disappearance were linear with time for 20 min and proportional to microsomal protein concentration. Apparent K_m and apparent V_{max} values were determined by graphical procedures using experimental

* In earlier literature the M5076 tumour is referred to as a carcinoma but it is now described as a sarcoma [13].

Table 1. Apparent K_m and V_{max} values for *N*-alkylmelamines and aminopyrine (AP)

Substrate	K_m (mM)	V_{max} (nmoles HCHO formed per mg microsomal protein or nmoles parent substrate metabolized per mg protein per min)
HMM (col.)	0.09 ± 0.01	2.6 ± 0.8
(g.l.c.)	0.08 ± 0.02	4.3 ± 0.09
HEM (g.l.c.)	0.15 ± 0.05	5.9 ± 1.2
PMM (col.)	0.23 ± 0.08	4.2 ± 0.6
tetraMM (col.)	0.91 ± 0.07	5.1 ± 1.5
triMM (col.)	1.7 ± 0.6	2.9 ± 0.9
2,2,4,4-Tetramethylmelamine (col.)	0.21 ± 0.03	1.8 ± 0.3
AP (col.)	0.8 ± 0.4	2.5 ± 0.7

* The values were obtained as the means of three different graphical methods (see Methods) and expressed as the mean ± S.D. of at least four experiments. In the colorimetric assay (col.) formaldehyde was measured, in the g.l.c. method disappearance of substrate was determined.

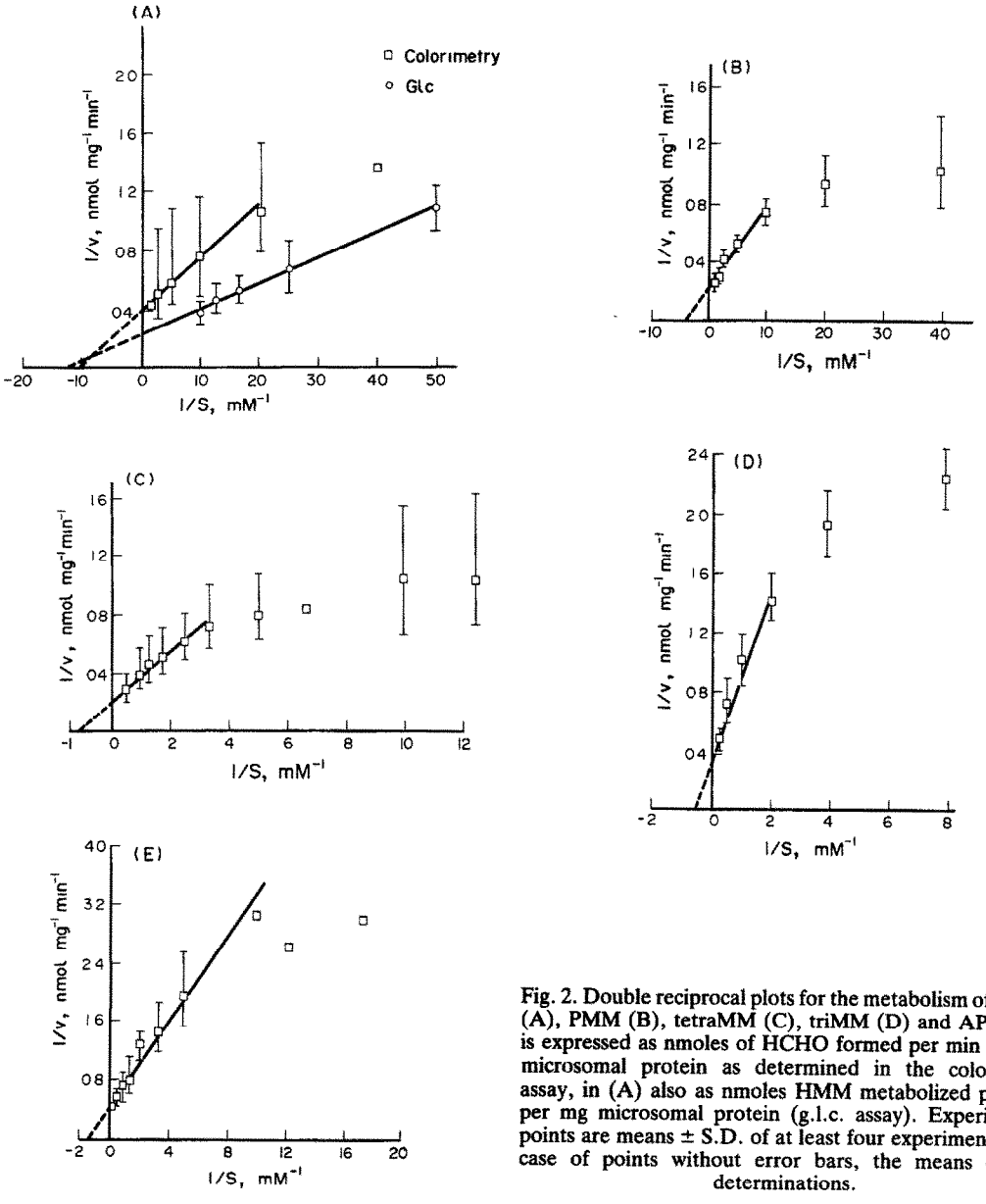

Fig. 2. Double reciprocal plots for the metabolism of HMM (A), PMM (B), tetraMM (C), triMM (D) and AP (E). v is expressed as nmoles of HCHO formed per min per mg microsomal protein as determined in the colormetric assay, in (A) also as nmoles HMM metabolized per min per mg microsomal protein (g.l.c. assay). Experimental points are means ± S.D. of at least four experiments or in case of points without error bars, the means of two determinations.

values for initial velocities (v) of the reactions (formaldehyde formation or disappearance of substrate) obtained for varying substrate concentrations S. The plots used were $1/v$ vs $1/S$ as devised by Lineweaver and Burk [22], S/v vs S as described by Hanes [23] and v vs v/S as described by Hofstee [24]. Intercepts, slopes and points of intersection were calculated from linear regressions for each experiment using the experimental points on the linear portions of the curves. The values obtained by the three different methods were not substantially different from each other and each individual constant is the mean of values computed by the three methods. Due to the heterogeneity of the enzyme source the values shown in Table 1 are of an indicative rather than a defining nature [25].

RESULTS AND DISCUSSION

Properties of hepatic HMM metabolizing enzymes

It is well established that HMM is extensively metabolized in the liver [26] and several studies have associated HMM metabolites with the drug's antineoplastic activity. Its major *in vitro* metabolite, *N*-hydroxymethyl-PMM [5] unlike HMM is directly cytotoxic [2–4]. However on the basis of available evidence, the possibility that the metabolic step which is crucial for the antitumour activity of HMM occurs extrahepatically, e.g. in the tumour, cannot be discounted. In fact a recent report shows unequivocally that HMM undergoes oxidative metabolism to PMM in L5178 Y cells [27]. Furthermore, labelled HMM on incubation with microsomes obtained from the M5076 sarcoma was covalently bound to microsomal protein [28], a finding which suggests a metabolic step transforming the inert HMM molecule into reactive electrophiles. Present evidence does not allow judgement as to the relative importance of hepatic and extrahepatic metabolism for the antitumour activity of HMM. However there is no doubt that hepatic *N*-demethylation leads ultimately to only weakly antineoplastic or inactive metabolites (tetraMM, triMM, dimethylmelamine, methylmelamine) and thus constitutes a drug detoxification. Figure 2 (A–E) shows the double reciprocal plots obtained from the kinetic analysis of the enzymatic *N*-demethylation of HMM, PMM, tetraMM, triMM and for comparison the plot for AP is also shown. Enzyme activity is expressed as the amount of formaldehyde and formaldehyde precursors [18] generated in mouse liver microsomal preparations. The Lineweaver–Burk plots are curvilinear concave downwards, and this shape has also been previously reported for the kinetic analysis of the metabolism of AP by rat liver microsomes [29]. The double reciprocal plots obtained by measuring the disappearance of HMM from the incubation mixture (Fig. 2A) or the appearance of PMM as a metabolite of HMM (not shown) by g.l.c. are similar to the curve representing the formation of the metabolite formaldehyde determined colorimetrically.

The linear portions of the double reciprocal plots consisting of values for high substrate concentrations were applied to compute apparent maximal velocity constants and apparent Michaelis constants. Two other plotting methods, v vs v/S and S/v vs S, were also employed. The constants are shown in Table 1. The apparent V_{max} values for the methylmelamines and AP are of a similar order of magnitude, and a decreased extent of methylation in the melamine molecule leads to a decreased affinity of the enzymes for the substrate. Rutty and Connors [6] also observed a relationship between the degree of metabolic *N*-demethylation (measured as total amount of formaldehyde formed in one hour) in rat liver microsomes and the number of methyl moieties on the melamine ring. It is however worth noting that in the order HMM, PMM and tetraMM, the decrease in affinity of the mouse liver enzymes for the substrates is accompanied by an increase in maximal velocities. The apparent K_m values for tetraMM and triMM (0.91 mM and 1.7 mM respectively) compared with those for HMM and PMM (0.09 mM and 0.23 mM respectively) may partly account for the long plasma elimination half lives of tetraMM and triMM as metabolites of HMM and PMM [10]: The low affinities of tetraMM and triMM for the mixed function oxygenases may result in their elimination via metabolism being much less prominent than that for HMM and PMM. A plot of initial velocities of metabolic formaldehyde generation against substrate concentration (Fig. 3) reveals that HMM—but not its *N*-demethylated derivatives or AP—appears to inhibit its own metabolism *in vitro* at substrate concentrations above 0.5 mM. It has been shown previously that HMM inhibits the microsomal *O*-demethylation of *p*-nitroanisole with an apparent K_i value of 0.8 mM [30]. Even though the drug concentrations for which these inhibitions were observed are far above drug levels reached during therapy this

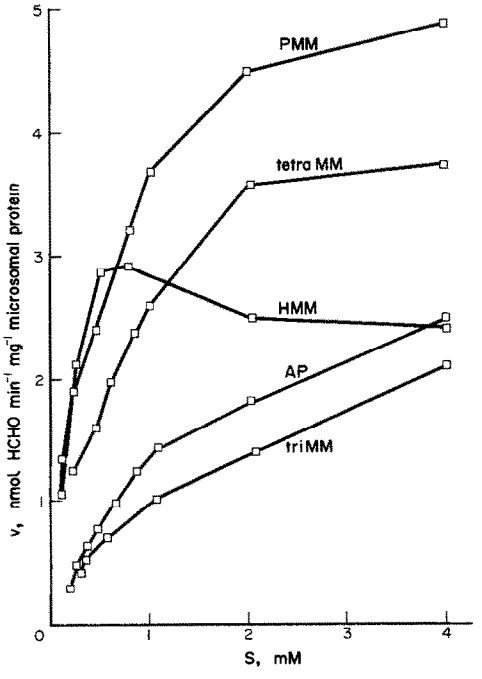

Fig. 3. *In vitro* metabolism of *N*-methyl melamines and AP. Initial velocities are plotted against substrate concentration. Points are the mean of at least four experiments.

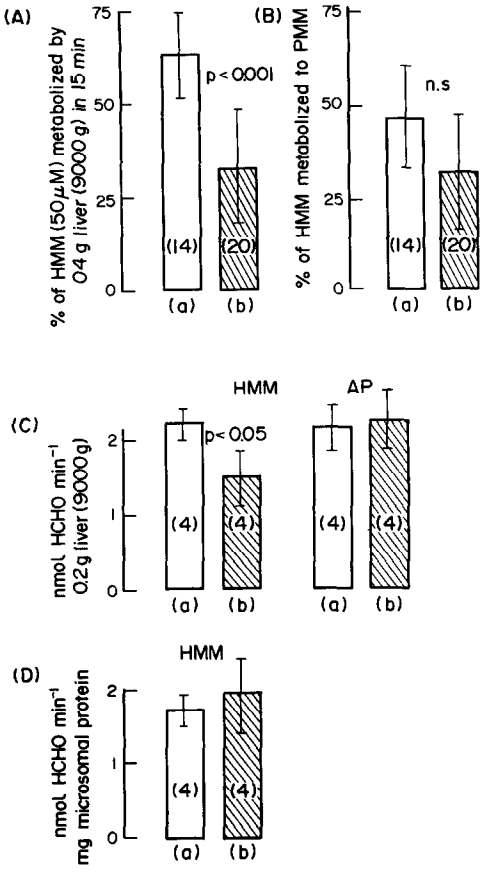

Fig. 4. The effect of administration of methyliodide on the *in vitro* metabolism of HMM and AP. (a) (open bars) indicates control values and (b) (hatched bars) values obtained with livers from treated animals. Numbers in brackets show numbers of experiments. (A) Measurement of disappearance of HMM by g.l.c., (B) determination of PMM by g.l.c., (C) and (D) colorimetric determination of formaldehyde as a metabolite of HMM and AP. (A), (B) and (C) were obtained with 9000 *g* supernatant preparations, (D) with liver microsomes. Only the values of (A) and (C) are significantly different from the respective controls.

finding is interesting because it is specifically associated with HMM.

It could be hypothesised that if metabolism renders the HMM molecule an antineoplastic species, there should be a specific difference in the biochemistry or chemistry of its *N*-demethylation as compared to the *N*-demethylation of non-cytotoxic *N*-methyl xenobiotics such as AP. As part of an effort to test this hypothesis we investigated the effect of the depletion of hepatic GSH on HMM metabolism. The oxidative *N*-demethylation of the carcinogen *N*-dimethylaminoazobenzene (DMAB) has recently been shown to be decreased on depletion of hepatic GSH pools [12]. This effect was specifically observed for DMAB *N*-demethylation whereas other pathways of oxidative metabolism which the DMAB molecule undergoes were not influenced. Figure 4 shows that administration of 75 mg/kg methyliodide which depleted mouse livers of GSH to 22 per cent of the normal level lead to a significant decrease in the rate of HMM *N*-demethylation in 9000 *g* liver preparations. After a 15 min incubation with 50 μM HMM 63.2 per cent was metabolised by livers of untreated mice whereas only 33.5 per cent was metabolized by livers of methyliodide pretreated mice (Fig. 4A). The appearance of PMM as a metabolite of HMM (Fig. 4B) and the metabolic generation of formaldehyde (Fig. 4C) were also reduced in incubation mixtures with 9000 *g* supernatant from livers of methyliodide pretreated mice (in case of the former (Fig. 4B) this reduction was not statistically significant). The *in vitro* *N*-demethylation of AP however was not changed by methyliodide administration (Fig. 4C) nor was the effect on HMM metabolism observed with isolated microsomes (Fig. 4D). There was also no significant effect of methyliodide administration on the disposition of HMM *in vivo* (Table 2). The influence of methyliodide pretreatment on the *in vitro* metabolism of HMM was not eliminated by protecting the animals with cysteine, a precursor of GSH, given 30 min before and shortly after administration of methyliodide. Furthermore this effect was not observed on treating mice with diethylmaleate which like methyliodide depletes hepatic GSH pools. So it is unlikely

Table 2. Disposition of HMM and its metabolite PMM in mice after administration of 38 mg/kg methyliodide 1 hr before 100 mg/kg HMM

	Control	Methyliodide treated animals
AUC HMM (μg/ml × min)	299 ± 72 (4)	388 ± 90 (4)
AUC PMM as metabolite of HMM (μg/ml × min)	406 ± 111 (4)	395 ± 100 (4)
Total radioactivity exhaled as $^{14}CO_2$* (% of injected dose)	23.5 ± 3.4 (5)	24.0 ± 2.0 (3)
$t_{\frac{1}{2}}$ of $^{14}CO_2$ appearance rate in the breath (min)	90.5 ± 5.6 (5)	116 ± 13 (3)

* Exhaled radioactivity and the half life of $^{14}CO_2$ appearance were determined as recently described [33].

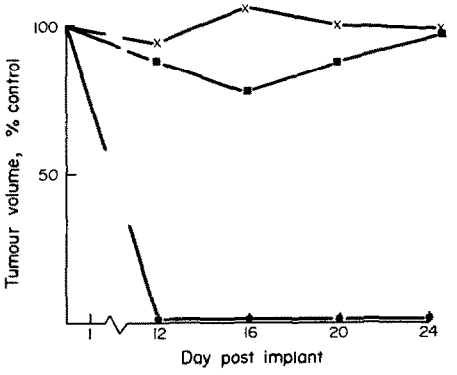

Fig. 5. Antineoplastic activity of HMM (●), melamine (×) and HEM (■) against the M5076 tumour. Drugs were administered i.p. daily for 17 days at 75 mg/kg (HMM) or 250 mg/kg (melamine and HEM). Details of tumour implantation and tumour volume measurement under Materials and Methods.

that the depletion of hepatic GSH pools causes the reduction of HMM *N*-demethylation. On addition to microsomal incubation mixtures with HMM 100 μM methyliodide decreased the metabolism of HMM by 25 per cent. So it is possible that methyliodide either inhibits HMM *N*-demethylation directly or affects an as yet unidentified cytosolic modulator of HMM metabolism *in vitro*. The observations described above for HMM were also made using PMM as substrate, but they are not shown here.

Antitumour testing and disposition of HEM

The murine M5076 reticulum cell sarcoma is one of the few experimental tumour models sensitive to HMM [31]. Figure 5 shows that it is also completely insensitive to HEM like the PC6A plasma cell tumour for which the structural requirement of *N*-methyl moieties in the melamine molecule has been reported previously [6]. HEM was less toxic than HMM to the host mouse with a LD_{10} of approximately seven times that of HMM (100 mg/kg given on 17 consecutive days), and a daily dose of 250 mg/kg for 17 days did not influence tumour growth. On the other hand 75 mg/kg HMM daily for 17 days led to a marked retardation of tumour development. Melamine, the N–H analogue of HMM (Fig. 1), was also without activity against the M5076 tumour (Fig. 5), its LD_{10} in the mouse was comparable to that of HEM.

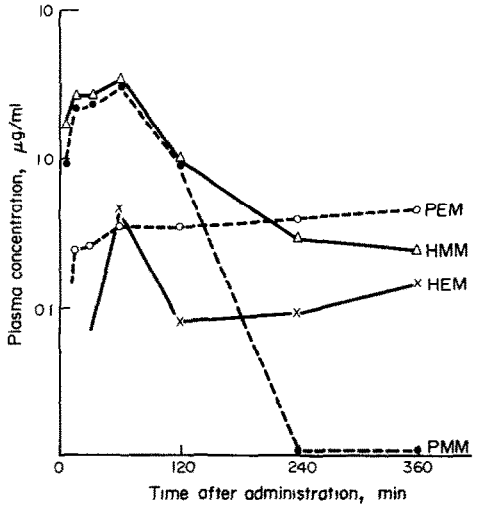

Fig. 6. Plasma levels of HEM and PEM after administration of 100 mg/kg HEM. Values are the mean of four mice for each time point. For comparison, the plasma concentrations of HMM and PMM after administration of 100 mg/kg HMM are shown as published before [10].

In contrast to the difference in antitumour activity *in vivo* HEM and HMM are equally toxic to PC6 cells in culture [2] and the rate of microsomal metabolism *in vitro* of HEM is similar to that of HMM (Table 1). To see whether there is a pharmacokinetic difference between the two agents which may account for the structural requirement for activity we studied the plasma disposition of HEM. Figure 6 shows the plasma concentrations of HEM and its *N*-dealkylated metabolite pentaethylmelamine (PEM) plotted against time after i.p. administration of 100 mg/kg HEM. For comparison the plasma concentrations of HMM and PMM after 100 mg/kg HMM as published before [10] are included in the graph. This figure shows that the drug levels achieved in the biophase do not approach the K_m values (Table 1) obtained using mouse liver microsomes. The K_m values can therefore only be interpreted as indicating a trend rather than reflecting the substrate concentration at the enzyme site *in vivo*. Plasma levels of HEM and PEM reach only a fraction of drug and metabolite levels obtained after HMM within three hours after administration (Fig. 6).

Substitution of the methyl moieties in the HMM molecule with ethyl groups imparts increased lipophilicity [32]. Therefore the difference in plasma levels between HMM and HEM may be due to the slower release of the less polar HEM from the acetone/oil injection vehicle. Alternatively, compared with HMM the HEM molecule may exhibit increased tissue affinity and a changed apparent volume of distribution. The observed difference in bioavailability between HMM and HEM indicates a pharmacokinetic basis for the requirement of *N*-methyl moieties for antitumour activity, i.e. it is conceivable to postulate that HEM is not an antitumour drug because it does not reach the tumour site at sufficient concentrations. There is no difference in the direct cytoxicity between HMM and HEM [2], but alkylmelamine cytotoxicity is directly related to the number of methyl moieties in the molecule. If one assumes that the HMM oxidizing enzymes in the tumour cell [27] are similar in their biochemical characteristics to the hepatic oxygenases investigated in this study, the lack of cytotoxicity of tetraMM, triMM and dimethylmelamine as compared to HMM and PMM may be due to insufficient rates of metabolic transformation to electrophilic *N*-hydroxymethyl derivatives or formaldehyde. So it is feasible to hypothesize that whereas the nature of the *N*-alkyl substituent determines the disposition and concentration at the target organ of the molecule via its physicochemical properties the number of alkyl groups determines the rate of formation of directly cytotoxic species. So a complex interplay of

pharmacokinetic and enzymatic factors, both influenced by structural alterations in the melamine molecule would determine the antitumour activity of this class of compounds. In which fashion the components of this interplay produce specificity for the tumour cell remains to be clarified in order to understand the mode of action of HMM.

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