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BIOCHEMICAL STUDIES ON THE ABILITY OF PENTAMETHYLMELAMINE TO INTERACT *IN VIVO* WITH DNA AND PROTEINS IN A SENSITIVE MURINE OVARIAN RETICULAR CELL SARCOMA

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Abstract—The metabolism of ^{14}C-PMM and its irreversible interaction with DNA and proteins were studied in M5076/73A reticular cell sarcoma, a murine solid tumor previously shown to be sensitive to the drug. Metabolism and irreversible binding were determined 0.25, 1, 8 and 104 hours after a single i.p. injection of radiolabelled PMM, tumor and liver macromolecular binding were compared with two differently ^{14}C-labelled PMM, i.e. ring- and methyl-PMM. Ring-PMM derived macromolecular binding appeared to have more relevance *in vivo* and had a similar time profile in both liver and tumor. Ring-PMM derived DNA binding was then related to metabolic steps between PMM and 2,2,4,6 TMM and 2,2,4,6 TMM itself and 2,4,6 TriMM.

PMM† is the first product of dealkylation of the antineoplastic drug HMM. It retains almost completely the therapeutic potential of the parent drug [1], being active on some experimental animal tumors [2, 3]. Unlike HMM, it is water-soluble, which makes it suitable for parenteral administration, though its therapeutic usefulness is severely limited by its neurotoxicity [4, 5, 6]. Pharmacokinetic studies have been made with both HMM and PMM in humans and laboratory animals [7–11]. The metabolism of both compounds is known and proceeds via a series of oxidative *N*-demethylation steps but their mechanism of action has not yet been clarified. HMM has been proposed as both a possible folic acid antagonist [12] and an indirect alkylating agent [13–15]. The latter suggestion has generally received more attention and the drug is believed to act through a covalent interaction with tumor macromolecules via a reactive metabolic product [16]. The same mechanism of action is postulated for PMM, though this molecule has been studied less. There is a report on the capacity of PMM to react *in vivo* with tumor DNA and RNA in a plasmocytoma ascitic animal model, and, when the reactivity of PMM was compared to that of HMM at the same lethal dose level [17] only minor quantitative differences were observed.

In an attempt to clarify the mode of action of PMM we determined PMM and some of its metabolites and investigated its covalent interaction with nucleic acids and proteins in liver and M5076/73A reticular cell sarcoma, a mouse solid tumor model which is reportedly sensitive to the drug [2]. We evaluated the reactivity of the ring skeleton containing metabolites and also the biochemical fate of one of the methyl groups of the molecule, in parallel experiments with ring and methyl labelled PMM (see Fig. 1). This paper is a continuation of previous work on the mechanism of action of HMM and PMM [16, 18, 19].

MATERIALS AND METHODS

Chemicals. Pentamethylmelamine-2,4,6-^{14}C hydrochloride (specific activity 12.6 mCi/mmole) and pentamethylmelamine-(*N*-methyl-^{14}C) hydrochloride (specific activity 15.8 mCi/mmole) were obtained from the Division of Cancer Treatment, NCI, NIH, Bethesda, MD, U.S.A. The radio-chemical purity of the two compounds was checked by thin layer chromatography on silica gel plates 60 F254 (Merck) in chloroform : methanol : ammonium hydroxide (19 : 1 : 0.1) and found to be 97% and 98% respectively.

Pentamethylmelamine; 2,2,4,6 tetramethylmelamine; 2,2,4,4 tetramethylmelamine; 2,4,6 trimethylmelamine; 2,2,4 trimethylmelamine and dimethylmelamime were also obtained from the Division of Cancer Treatment, NCI, Bethesda, MD. All other reagents were of the purest grade commercially available and were used with no further purification.

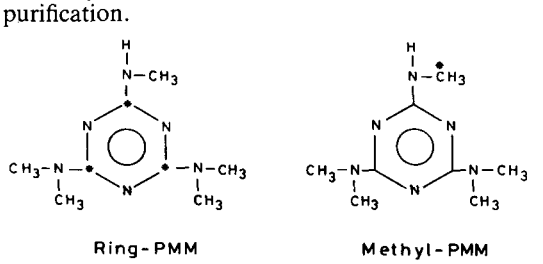

Fig. 1. Chemical structure of the two differently labelled ^{14}C-PMM. The asterisks indicate the radiolabelled carbon atom.

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† Abbreviations: PMM, pentamethylmelamine; 2,2,4,6 TMM, 2,2,4,6 tetramethylmelamine; 2,2,4,4 TMM, 2,2,4,4 tetramethylmelamine; 2,4,6 TriMM, 2,4,6 trimethylmelamine; 2,2,4 TriMM, 2,2,4 trimethylmelamine; DMM, dimethylmelamine.

Animal treatment. Female C57B1/6J mice weighing 20 ± 2 g were intramuscularly injected with 0.5×10^6 cells of M5076/73A ovarian cancer. Twenty days after inoculation, when the animals weighed 24 ± 3 g and the tumor weighed 4.2 ± 0.4 g, animals were injected i.p. with 50 mg/kg of isotopically diluted ^{14}C-PMM hydrochloride dissolved in 0.2 ml of saline. ^{14}C-PMM labelled at two different positions was used, i.e. PMM-(*N*-methyl-^{14}C) hydrochloride (methyl-PMM) and PMM-2,4,6-^{14}C hydrochloride (ring-PMM). In the same experiment, the same day, half the animals were treated with methyl-PMM (30×10^6 DPM/animal diluted with carrier PMM to a specific activity of 3.3 mCi/mmole to obtain the desired dose) and the other half were given ring-PMM (30×10^6 DPM/animal diluted with carrier PMM to a specific activity of 3.0 mCi/mmole to obtain the desired dose).

At 0.25, 1, 8 and 104 hours 6 animals per time point for each experimental group were killed by bleeding from the right eye, and the liver and viable part of the tumor were excised. The tissues were immediately frozen on dry ice and stored at −18° until analyzed.

Determination of total radioactivity (PMM + metabolites) in liver and tumor. The two tissues were homogenized in 10 mM Tris–HCl buffer pH 7.8 containing 75 mM NaCl and 10 mM Na_2 EDTA (1–3 weight/volume) using a Potter–Elvejehm homogenizer with a Teflon pestle; 100 μl were collected and dissolved in 0.5 ml of Soluene R-350 and transferred into counting vials containing 10 ml of PPO-POPOP solution in toluene. Determinations were made in duplicate. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter. The values were corrected for quenching by the external standardization method.

Determination of PMM and metabolites in liver and tumor. PMM and its metabolites were determined in tumor and liver after HPLC separation. To 0.4 ml of liver or tumor homogenate (1–3 w/v) 50 μl of NaOH 1 M were added. The samples (pH ≃ 11.5) were then extracted twice with 4 ml ethyl-acetate. The organic phase was dried under a gentle stream of nitrogen and redissolved in 100 μl of 100 μg/ml solution of PMM and metabolites; 25 μl of this solution were injected into a Waters model 6000 A HPLC equipped with a prepacked phenyl Bondapak column (Waters Ass.) and a 254 nm adsorption detector. PMM and metabolites were separated with a linear gradient from 35% A (0.005 M 1-octane sulphonic acid in methanol pH 5), 65% B (0.005 M 1-octane sulphonic acid in water pH 5) to 45% A, 55% B in 10 min.

Fractions corresponding to PMM and metabolite peaks were collected in glass vials and lyophilized. After lyophilization samples were redissolved in 100 μl of methanol and 10 ml of Filter Count (Packard) scintillation solution and radioactivity was measured as described in the previous section.

Due to the acidic pH, our chromatographic method is not able to determine PMM methylol derivatives, but separate experiments with methods suitable to detect *N*-methylol melamines have never been successful in this respect.

Covalent binding to DNA. The DNA was extracted from liver and tumor by a slight modification of the hydroxyl hapatite method of Viviani and Lutz [20], the main difference consisting in the fact that a batch procedure rather than a column procedure was followed. In brief, tissues were homogenized in 3 vol of 10 mM Tris–HCl buffer pH 7.8 containing 75 mM NaCl and 10 mM Na_2 EDTA using a Potter–Elvejehm homogenizer with a Teflon pestle. A solution of Nonidet P-40 2% in water was added to the homogenate to a final concentration of 0.2%. The mixture was left on ice for 15 min and centrifuged in a Beckman refrigerated J-20 centrifuge at 700 *g* for 5 min. The supernatant was collected and subsequently processed for the determination of radioactivity bound to proteins (see "covalent binding to proteins" section below). The pellet was resuspended, using a vortex, in the same buffer as before in the absence of Nonidet P-40 and centrifuged at 3500 *g* at 4° for 5 min. The supernatant was discarded, the pellet resuspended using a vortex in 25 ml of 0.24 M sodium phosphate buffer pH 6.8 containing 8M urea, 10 mM Na_2 EDTA and 1% SDS. The suspension thus obtained was more evenly homogenized with a Ika-Werk Ultraturrax set at medium speed in two 15-sec bursts, with rapid cooling of 1 min at 0° in between to prevent the sample foaming. To this aqueous suspension 10 ml of phenol mixture (phenol–chloroform–isoamyl alcohol 25–24–1) were added and the two phases were evenly emulsified by shaking for 10 min then centrifuged at 20,000 *g* at 25° for 15 min. The aqueous phase containing nucleic acids was removed and further extracted with 10 ml of phenol mixture as above. The aqueous phase was extracted twice with 25 ml of diethylether to remove the excess phenol and left in the dark at room temperature overnight. The nucleic acids solution was added to a suspension of hydroxylhapatite (DNA grade-Biorad Richmond, U.S.A.) left overnight to swell in 6 vol of 8 M urea in 0.24 M sodium phosphate buffer pH 6.8. The suspension was gently rolled for 2 hr to permit the adsorption of DNA on the hydroxylhapatite matrix, the hydroxylhapatite was rapidly spun down by a table centrifuge and the supernatant was discarded. The gel was gently resuspended using a glass rod in 40 ml of 8 M urea in 0.24 M sodium phosphate buffer pH 6.8 and rolled again for 15 min. This step was repeated twice, after which no more adsorption at 260 nm was observed in the washings. The gel was washed free of urea by two consecutive resuspensions in 0.014 M sodium phosphate buffer pH 6.8 as above. The DNA was eluted from hydroxylhapatite by two resuspensions in 10 ml each of 0.48 M phosphate buffer pH 6.8. The DNA was dialysed against 2500 vol of 0.2 M NaCl for 16 hr then precipitated with 2 vol of ethanol at −18° for 18 hr. The ratio of hydroxyl haptatite to tissue used for the purification of DNA was 2 g for each gram of liver and 3 g for each gram of tumor. In our hands this method, as judged from spectrometric analysis, yielded highly purified DNA with 260/280 Δ0 ratios of 2.0–2.1 and 260/230 ratios of 2.2–2.4. These compare well with those of Viviani and Lutz [20] and others [21, 22].

The recovery of DNA from tumor tissue was: 1.5 ± 0.1; 1.6 ± 0.1; 3.3 ± 0.3; 4.2 ± 0.8 whereas the recovery from liver was: 0.8 ± 0.1; 0.8 ± 0.1;

1.1 ± 0.2; 1.5 ± 0.2 at 0.25, 1, 8, 104 hr respectively. Values are expressed as mean ± S.E. (N = 6).

For the determination of radioactivity bound to DNA, the nucleic acid was dissolved in 1 ml of 0.5 M perchloric acid at 70° for 15 min, 800 μl were added to a 10 ml glass scintillation vial containing 10 ml of Lumagel (Lumac AG, Basel, Switzerland) and counted as described in the previous section. Two tissue samples containing no radioactivity were always run in parallel throughout the whole procedure to determine background counts and to rule out possible radioactive contamination. The background counts always ranged between 16 and 18 CPM in our experimental conditions. Experiments carried out adding radiolabelled ^{14}C ring PMM to tumor homogenate followed by immediate extion of DNA showed that DNA associated radioactivity was not different from that of tumor homogenate added with cold PMM.

The remaining 200 μl were utilized for the determination of DNA content by the method of Burton [23] using herring sperm DNA as standard. The DNA utilized for our determinations was in the range of 3 to 6 mg for tumor and 1 to 2 mg for liver.

Covalent binding to proteins. Two milliliters of 700 *g* centrifugation supernatant were mixed with 5 ml of acetone, the precipitate was centrifuged at 3000 *g* for 15 min and washed 5 times with 5 ml of methanol until no further radioactivity could be extracted. The precipitate was washed once more with 5 ml of acetone and dried overnight. The pellet was resuspended, using a tight Potter Elvejehm homogenizer with a glass pestle, in 5 ml of water. From the resulting even suspension 1 ml was taken, centrifuged and dissolved in 1 ml Lumasolve (Lumac AG, Basel, Switzerland), transferred to a glass scintillation vial containing 10 ml of Lipoluma (Lumac AG, Basel, Switzerland) and counted as described. Another aliquot was used for the determination of proteins according to Lowry *et al.* [24] using bovine serum albumin as standard.

This protein fraction, chosen for practical reasons, is a good representation of the total protein binding from which it does not differ by more than 12% (data not shown). It represents 88 ± 1% of total proteins in liver and 82 ± 2% in tumor tissue (mean ± S.E. N = 3). It is 3.9 ± 0.4% contaminated with RNA and less than 0.5% with DNA in liver and 1.8 ± 0.1% contaminated with RNA and less than 0.5% with DNA in tumor tissue as determined respectively by the methods of Ceriotti [25] and Burton [23]. Again, all results are expressed as the mean ± S.E. of three determinations. Values of protein binding in both tissues should not be grossly distorted by this RNA contamination because the *in vivo* reactivity of PMM towards cellular RNA has been shown to be approximately twice that to DNA (though allowance must be made for the different experimental conditions) [17].

RESULTS

Figure 2 (panels a and b) shows the level of total radioactivity in liver and tumor tissue after single i.p. injections of ^{14}C-ring and ^{14}C-methyl PMM at four different times. The highest level of radioactivity was almost three times in liver as in tumor but the difference tended to decrease as time passed, to 1.5 at 1 hr and much less at 8 and 104 hr. This naturally implies a higher rate of clearance of PMM and metabolites in liver than in tumor. Although there was no difference in the initial level of radioactivity in both tissues with the two different labels (panel b versus panel a), the clearance of methyl label at 1 hr was much higher. This difference between the two labels can be explained on the basis of DMM and possibly of monomethylmelamine (the latter was not determined in our experimental conditions but is likely to amount to the difference between total radioactivity and the sum of all metabolites determined at 15 min and 1 hr) which had no methyl label and whose levels were substantial at 1 hour. By 8 hours almost all the radioactivity has disappeared.

Table 1 shows the levels of PMM and all the known metabolites except for monomethylmelamine in liver, and Table 2 shows the tumor levels. The disappearance of the various metabolites in both tissues followed a very similar pattern, PMM always peaking at 15 min, with all the metabolites, starting from 2,2,4,4 TMM, markedly decreasing in concentration between 1 and 8 hr. PMM and 2,2,4,6 TMM already showed considerable clearance between 15 min and 1 hr. The only two metabolites whose concentration increased between 15 min and 1 hr were 2,4,6 TriMM and DMM in tumor, whereas only DMM showed this trend in liver.

It is worth noticing that the sum of all metabolites at 15 min and 1 hr corresponds to almost 90% of total radioactivity in liver and almost 100% in tumor (see Fig. 2, panel a).

The binding of PMM reactive metabolites to protein and DNA, as determined by the ring labelled molecule, is presented in Fig. 3 (panel a for liver and panel b for tumor). In both cases binding to proteins was much higher than binding to DNA, its ratio ranging from 4 to 8 at the different times in liver and from almost 3 to 5 in tumor. The profile of the protein binding curve seems almost the same in the two tissues though in tumor the peak, is shifted towards eight hours whilst the level of binding in liver tends to be maximal already at 1 hr. The DNA binding profile was very similar in liver and tumor, the highest level of binding being at 1 hr when its absolute value was equal in the two tissues. The peak value of binding was however, reached faster in liver than in tumor because at 15 min the degree of binding was double in the liver.

Figure 4 panel a and panel b shows the level of radioactivity irreversibly associated with proteins and DNA in liver and tumor after the administration of methyl-PMM. The curves of protein and DNA associated radioactivity are very different in the two tissues, with the amount of radiolabel in DNA being greater than in proteins in M5076/73A at all times except 15 min, whereas in liver the only time at which labelled nucleic acid was in excess was 104 hr. Some other points are worth noticing: (a) Protein associated radioactivity was higher at all time points in liver than in tumor though the dynamics of this parameter in the two tissues was very similar; (b) except at 15 min, exactly the reverse holds true for DNA associated radioactivity; (c) there were no

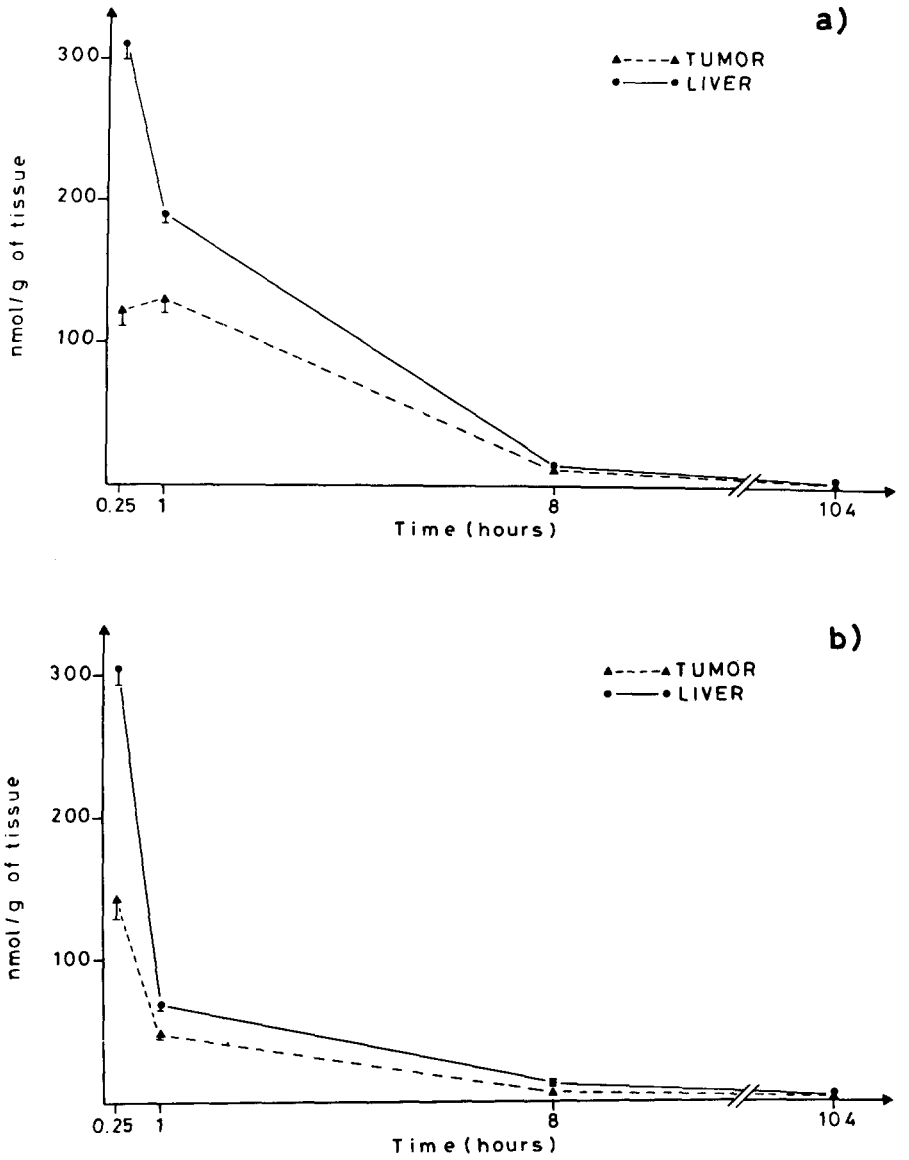

Fig. 2. (a) Total radioactivity in liver and tumor tissue after a single i.p. injection of 50 mg/kg ring-PMM at four different times. Results are expressed as mean ± S.E. of six animals. (b) Total radioactivity in liver and tumor tissue after a single i.p. injection of 50 mg/kg methyl-PMM at four different times. Results are expressed as mean ± S.E. of six animals.

Table 1. Pentamethylmelamine and metabolites in liver (nm/g of tissue)

Time (hr)	PMM	2,2,4,6-TMM	2,2,4,4-TMM	2,2,4-TriMM	2,4,6-TriMM	DMM
0.25	50.7 ± 5.2	112.2 ± 10.9	13.4 ± 3.5	21.7 ± 2.1	79.2 ± 8.1	17.2 ± 0.9
1	12.0 ± 1.3	13.4 ± 4.3	8.7 ± 0.9	17.3 ± 2.1	89.0 ± 18.6	39.3 ± 2.5
8	4.9 ± 1.3	8.2 ± 3.2	9.9 ± 6.2	4.7 ± 1.6	5.8 ± 3.5	6.8 ± 2.7
104	9.4 ± 3.5	5.8 ± 2.7	13.6 ± 3.4	13.4 ± 5.1	9.0 ± 4.0	8.7 ± 2.3

Results after a single i.p. injection of 50 mg/kg ring-PMM. Results are mean ± S.E. of six animals; samples were run in duplicate.

Table 2. Pentamethylmelamine and metabolites in tumor (nm/g of tissue)

Time (hr)	PMM	2,2,4,6-TMM	2,2,4,4-TMM	2,2,4-TriMM	2,4,6-TriMM	DMM
0.25	39.2 ± 2.6	44.9 ± 4.8	8.9 ± 1.2	15.7 ± 1.0	23.7 ± 1.7	11.2 ± 0.8
1	9.0 ± 1.6	13.5 ± 2.6	10.5 ± 2.1	19.2 ± 1.7	59.4 ± 4.1	37.9 ± 4.9
8	1.2 ± 0.4	1.1 ± 0.2	1.2 ± 0.3	0.9 ± 0.1	1.5 ± 0.1	1.8 ± 0.2
104	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.2 ± 0.3

Results after a single i.p. injection of 50 mg/kg ring-PMM. Results are mean ± S.E. of six animals; samples were run in duplicate.

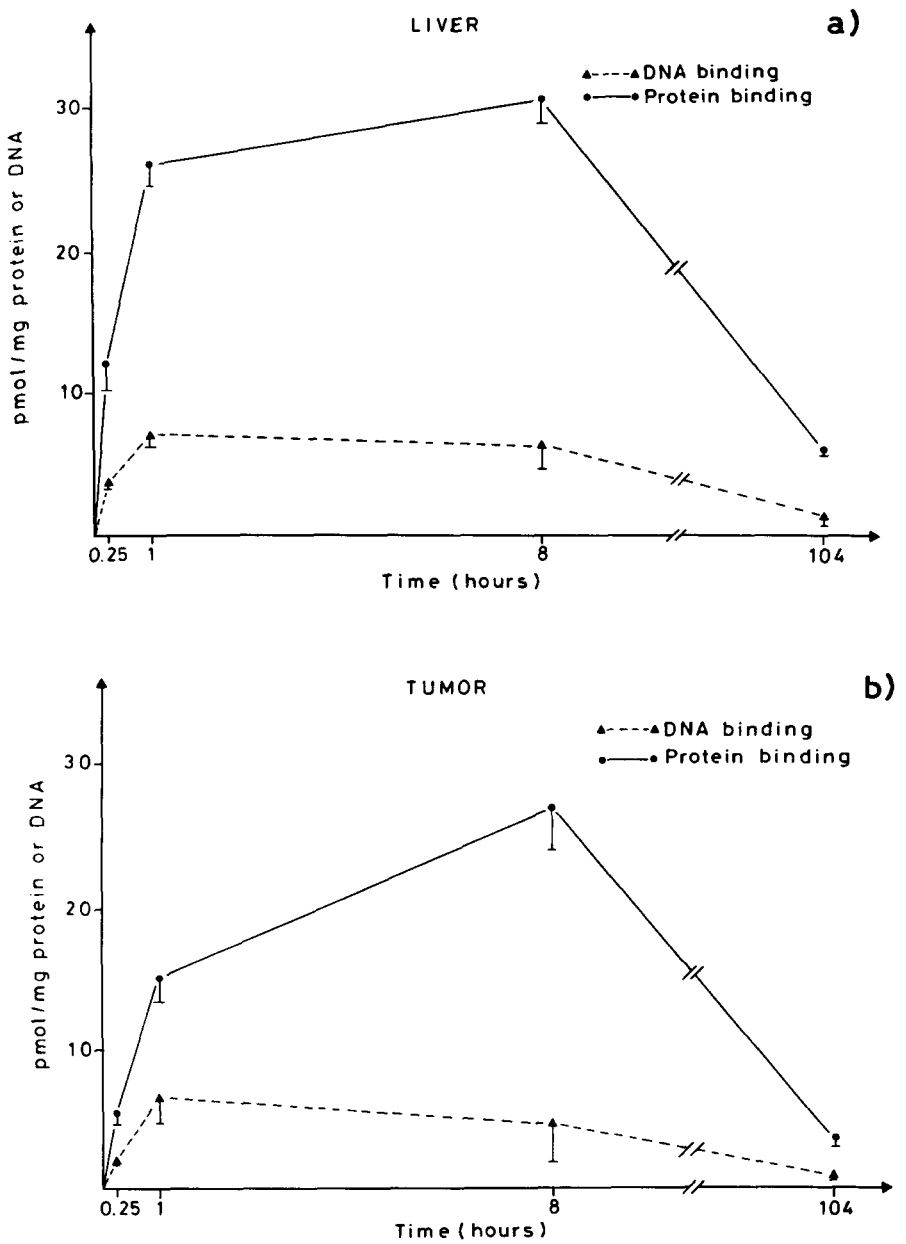

Fig. 3. (a) DNA and protein covalent binding in liver after 50 mg/kg, ring-PMM. Results are mean ± S.E. of six animals. (b) DNA and protein covalent binding in tumor after 50 mg/kg ring-PMM. Results are mean ± S.E. of six animals.

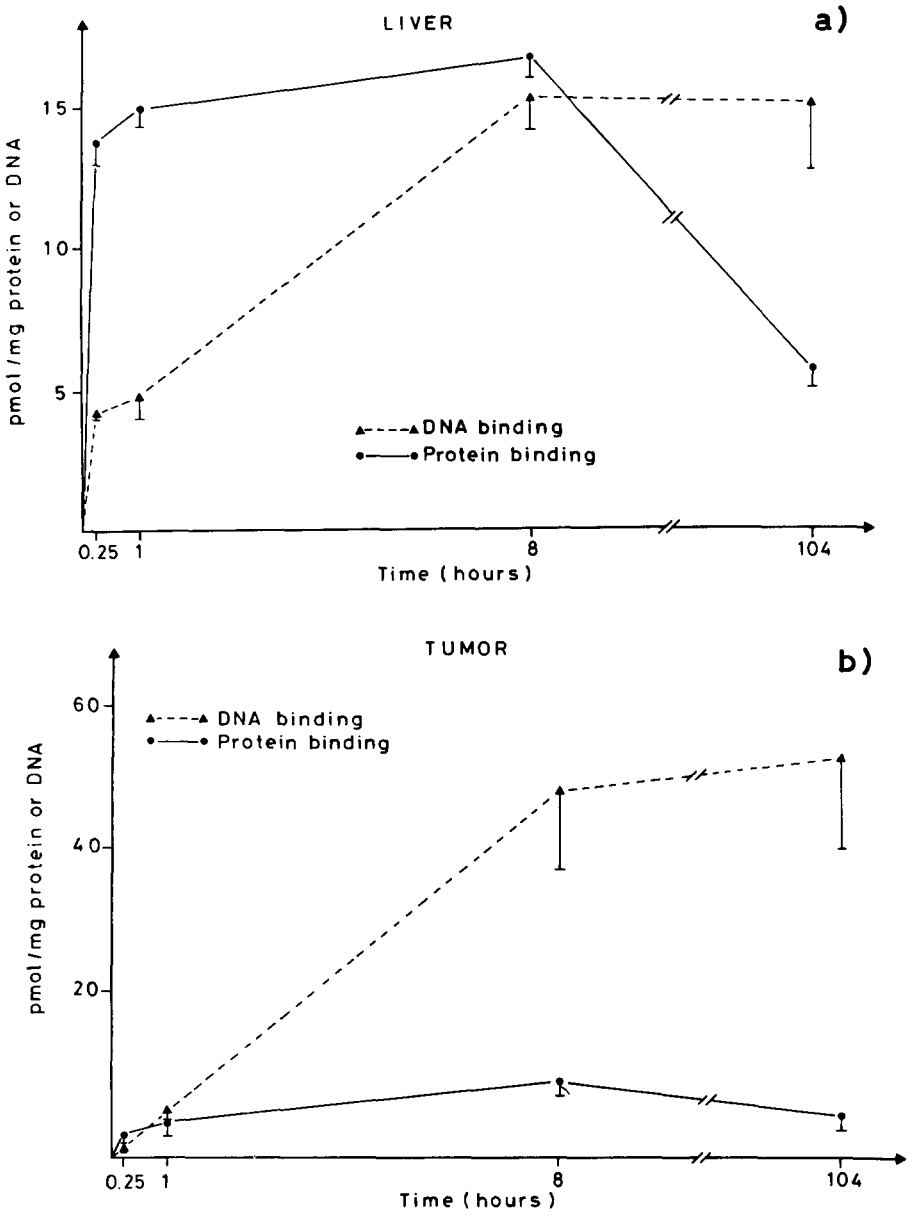

Fig. 4. (a) DNA and protein covalent binding in liver after 50 mg/kg methyl-PMM. Results are mean ± S.E. of six animals. (b) DNA and protein covalent binding in tumor after 50 mg/kg methyl-PMM. Results are mean ± S.E. of six animals.

major quantitative differences in protein radioactivity in tumor and liver at all four times, and in fact a plateau was almost attained after 15 min, but little clearance could be observed between 8 and 104 hr in tumor tissue, whereas in the same time span the level in liver fell to virtually one third; (d) in both liver and tumor, DNA associated radioactivity tended to reach a plateau after 104 hr, the greatest increase in value is always being observed between 1 and 8 hr. In any case irreversibly associated methyl-derived radioactivity should be regarded as a gross underestimation of reality in our experimental conditions because of the asymmetric labelling of methyl-PMM.

DISCUSSION

The metabolism, DNA and protein binding of PMM in liver and tumor tissue were determined after a single i.p. injection of radiolabelled PMM at a dose which proved active in this experimental model (unpublished results). Macromolecular binding was compared in liver and tumor, i.e. respectively in a non-target tissue with high metabolic potential and in the drug target tissue.

Comparisons were also made between two different radiolabels of PMM—ring and methyl—to establish the fate of these two parts of the molecule as regards their potential to associate irreversibly

with DNA and proteins. With ring-PMM, which should show up the most important metabolites from the point of view of macromolecular reactivity and possibly cytotoxicity [14, 18], no great differences were observed in the absolute level or the dynamics of DNA binding in tumor and liver though the exposure of the two tissues to the parent drug and metabolites was higher in liver than in tumor (see Fig. 2 and Tables 1 and 2). DNA binding exposure versus PMM and metabolites exposure for the two different tissues cannot be calculated with precision. However, a gross index may be established by the ratio between the highest level of DNA bound radioactivity and the highest level of total radioactivity in liver and tumor (compare Fig. 3 and Fig. 2 panel a and Fig. 3 and Fig. 2 panel b). This is three times higher in liver than in tumor tissue, probably indicating that M5076/73A cells are more exposed to DNA damage than their hepatic counterparts. The ratio is closer for protein binding (approximately 2 to 1 in favour of tumor), but the largest differences are present at early times and tend to become smaller or disappear altogether at 8 and 104 hr. The disappearance of radioactivity in tumor and liver DNA between 8 and 104 hr can theoretically be attributed to three different mechanisms: (a) isotopic dilution due to DNA synthesis; (b) cell distruction; (c) real DNA repair.

If uniform labelling of the cell population in both liver and tumor is assumed, points (a) and (b) seem unlikely because the difference in the level of DNA binding are too large between 8 and 104 hr to be entirely due to these two processes, which would in fact call for very great changes in tumor and liver mass between these two times. Thus if repair mechanisms are taken into consideration there is no substantial difference in the ability of either tissue to respond to DNA injury. It is further more clear that the same holds true for protein binding.

The very great differences between methyl- and ring-PMM for DNA associated radioactivity have, in our opinion, only one explanation: this phenomenon is due to incorporation, not to binding. In fact, the specific activity of DNA is higher in tumor than in liver, i.e. in a tissue where DNA synthesis is higher but metabolism should be much reduced [16]. Hence, to explain the five-fold difference migration of formaldehyde liberated during PMM biotransformation from liver or possibly other sites of metabolism to tumor tissue, selective concentration in the tumor or highest affinity for tumor DNA should be presumed. All this seems unlikely since formaldehyde itself is highly reactive towards protein nucleophilic centers [14] which should prevent its migration, moreover there are at least two metabolic pathways by which formaldehyde could get incorporated into DNA, i.e. via oxidation to formate or carbon dioxide and subsequent entry into the tetrahydrofolate pool or pyrimidine pool respectively [26, 27]. Furthermore, the *in vitro* reactivity to DNA of methyllabelled HMM and ring-labelled HMM did not differ [14], suggesting involvement of the same metabolite(s) involved in this interaction, and poor reactivity of nascent formaldehyde to DNA, but high reactivity towards microsomal proteins. If this also holds true for PMM this would further strengthen our original assumption.

DNA and protein associated radioactivity after administration of ^{14}C-ring labelled PMM should be due to covalent interaction even though on the basis of our experiments we cannot exclude the possibility that a minor presently unknown metabolic pathway may result in chemical species which could be incorporated into cellular macromolecules. However, this does not seem to be the case since as shown for tumor DNA binding no increase in DNA associated radioactivity is observed between 1 and 8 hr at which time DNA synthesis is increased (see "Materials and Methods" section under "Covalent binding to DNA" heading) while PMM and all metabolites are still well represented.

As to the molecular species involved in DNA and protein binding, some observations seem to be of interest. Ring-PMM DNA binding increases in both liver and tumor tissue between 15 min and 1 hr, during which time the only metabolites that disappear consistently are PMM and 2,2,4,6 TMM. Thus the reactive species responsible for DNA binding should be sought in the metabolic step between PMM and 2,2,4,6 TMM or possibly between 2,2,4,6 TMM and 2,4,6 TriMM. In fact the concentrations of all the other metabolites decrease significantly between 1 and 8 hr during which time binding does not increase. It is thus suggested that methylol derivatives of PMM and TMM might be the molecular species involved in binding, and this is supported by recent *in vitro* findings [14].

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