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# QUANTITATIVE ASSESSMENT OF THE BINDING OF ACETAMINOPHEN METABOLITES TO MOUSE LIVER MICROSOMAL PHOSPHOLIPID

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**Abstract**—Phospholipids were quantitatively extracted from microsomes and separated by an h.p.l.c. gradient system with a solvent mixture of *n*-hexane/*n*-propanol/water/acetic acid. In a model reaction using horseradish peroxidase/$H_2O_2$ in order to activate acetaminophen and inactivated microsomes as target, a covalent binding of 10 nmol drug metabolite per mg microsomal lipid was found. In isolated intact microsomes from methylcholanthrene-pretreated male albino mice, a binding of 0.1 nmol acetaminophen metabolite per mg phospholipid was determined while the binding of metabolites to protein amounted to 3 nmol/mg. The results demonstrate that in mouse liver microsomes metabolizing acetaminophen, about one out of $10^4$ phospholipid molecules is modified.

Acetaminophen, although a safe drug in clinical use [1], produces severe liver damage in man when overdosed. In laboratory animals the drug is frequently used as model for chemically induced hepatic lesions. The molecular mechanism by which the metabolic competence of the cell is ultimately destroyed is still open to discussion [2]. Also, proposals as to the chemical nature of the primary reactive metabolite(s) include benzoimidoquinone [3, 4], *N*-OH-derivative [5] or semiquinone structures [6]. In early investigations, the phenomenon of the covalent binding of reactive metabolites of the drug in microsomes was recognized as well as the significance of endogeneous glutathione as a protective factor [7]. However, several inconsistencies prompted us [8] and others [9] to suggest that lipid peroxidation by reactive oxygen species derived from the monooxygenase reaction might be the primary cytotoxic event of the drug's adverse effects. In a series of investigations, we studied this concept *in vivo* [10], *in vitro* [11] and in the perfused organ [12] and showed that lipid peroxidation was an early, dose-dependent and therefore probably causal event in the sequence leading to liver destruction. However, we were not able to differentiate between membrane lipid deterioration induced by attack of oxygen radicals to polyunsaturated fatty acids or a direct reaction of organic, i.e. drug radicals/reactive species, or both.

In this report we demonstrate that such an AAP metabolite binds to microsomal phospholipids, estimate its degree and discuss its significance for the mechanism of the primary lesion induced by the metabolism of the drug.

Abbreviations: AAP acetaminophen (paracetamol); h.p.l.c., high-performance liquid chromatography; HRP, horseradish peroxidase; PC, phosphatidyl-choline; PE, phsophatidyl-ethanolamine; PI, phosphatidyl-inositol; PS, phosphatidyl-serine; PL, phospholipid.

## MATERIALS AND METHODS

*Animals*. Male albino mice were kept on a commercial diet for at least three weeks (Botzenhardt KG, Kempten, F.R.G., diet H-1003) with free access to food and tap water until 24 hr before the experiment. The mice were pretreated for three consecutive days by intraperitoneal injection of 20 mg/kg body weight methylcholanthrene in sesame-oil.

*Chemicals*. For h.p.l.c. analysis an SP 8700 system with integrator SP 4100 was used. H.p.l.c. grade solvents were from Promochem (Wesel, F.R.G.). HRP (grade I, specific activity 250 U/mg), glucose-oxidase (grade I, specific activity 250 U/mg), NADPH and isocitrate dehydrogenase (grade I, specific activity 3 U/mg) were from Boehringer (Mannheim, F.R.G.). 3,5-[$^{14}$C]AAP (25 mCi/mmole, radiochemical purity 99% checked by h.p.l.c. in the system of Ref. [15]) was from Amersham Buchler (Braunschweig, F.R.G.). 3-Methylcholanthrene, AAP, DL-isocitrate and phospholipids (PC, PS, PI, phosphatidic acid, PG, PE) were from Sigma (München, F.R.G.).

*Assays*. Liver microsomes were prepared according to reference [13] in 0.25 mM sucrose. They were washed twice in 0.15 mM Tris buffer (pH = 8) and finally resuspended in 0.1 mM potassium phosphate buffer (pH = 7.4) containing 50 mM KCl. Microsomes were kept stored in this solution at −80° no longer than for 6 days. This did not result in measurable variations in enzymatic activities as assessed by ethoxycoumarin dealkylation. The microsomes were warmed prior to use and then incubated in the presence of AAP as described. For control incubations, microsomes were heat-inactivated at 70° for 5 min or at 95° for 3 min.

The protein content of the dried lipid extracts, in addition to a survey of the elution profile at 280 nm, was checked by the method of Coomassie blue bind-

Table 1. Binding of acetaminophen metabolites to phospholipid or protein of liver microsomes from male mice. Incubations were conducted at 37° for 15 min in 100 mM potassium phosphate buffer pH = 7.4; containing 1 mg/ml microsomal protein and 1 mM [$^{14}$C]AAP; controls: boiled microsomes

| Pretreatment | System | AAP bound to protein [nmol/mg] | AAP bound to lipid* [nmol/mg] |
|---|---|---|---|
| none | NADPH | 1.7 | n.d. |
| methylcholanthrene | NADPH | 2.9 | 0.1 |
| none | HRP/$H_2O_2$ | 78 | 10.3 |

* Calculated from phosphate analysis; n.d. = not detectable.

ing to protein [14]. Under our conditions, calibrated with bovine serum albumin, 1 $\mu$g of protein could be accurately determined.

Protein binding was assessed by incubating 1 mM [$^{14}$C]AAP (Amersham), sp. act. 0.5 mCi/mmole up to 10 mCi/mmole with 1 mg/ml microsomal protein for 10–30 min at 37°. The following NADPH-regenerating system was used: 7 mM isocitrate, 3.3 mM $MgCl_2$ and 0.5 U/ml isocitrate-dehydrogenase. In the case of HRP-activation, AAP was preincubated for 5 min with 10 mM glucose and 0.05 U/ml HRP at 25°, then 0.5 U/ml glucose oxidase were added in order to provide a continous $H_2O_2$ formation.

*Lipid binding.* 0.1 mole/l potassium-phosphate buffer (pH 7.4) and 0.8 or 1 mM [$^{14}$C]AAP (10 mCi/mmol) were used as standard incubations, with the following specific additional conditions: (i) Suspensions of PE or PC (1 mg/ml) were incubated for 10 min at 25° with the HRP-system where glucose/glucose oxidase was used in order to generate $H_2O_2$ [19]. (ii) Inactivated microsomes (10 mg/ml) were incubated with the HRP/glucose oxidase system in analogous assays as used for covalent protein binding. (iii) When AAP-metabolites were generated via NADPH-dependent monooxygenase pathways followed by assessment of lipid binding, incubation volumes amounted up to 5 ml in the presence of 0.8 mmole/l [$^{14}$C]AAP with a specific activity of 10 mCi/mmole.

In order to minimize the water content of microsomal suspensions, the incubates were concentrated before extraction by ultracentrifugation (60 min with 40,000 *g* at 0°) in the presence of 0.02% $NaN_3$ and 0.05% 2,6-di-tert.-butyl-4-methylphenol (BHT). The resulting pellet was suspended in potassium-phosphate-buffer (100 mmoles/1, pH = 7.4) and extracted by the method of Hara and Radin [16]. The following h.p.l.c. gradient-system was established on a LiChrospher Si 100, 10 $\mu$m silicagel-column (250 mm length) from Bischoff (Berlin/West):

(A) *n*-hexane/*4n*-propanol/water/acetic acid = 900/1200/30/0.
(B) *n*-hexane/*n*-propanol/water/acetic acid = 750/1000/93.8/0.25.
(C) *n*-hexane/*n*-propanol/water/acetic acit = 750/1000/164.1/0.25.

Flow rate: 1.5 ml/min, $t = 25°$, PL-extract sample size up to 2 ml solved in component A. After 5 min. with (A), isocratic, a linear gradient from B to C was run terminated by an reverse gradient back to A within 5 min and a conditioning phase of about 10 min. Phospholipids were detected at 206 nm. Protein content was followed at 280 nm. Fractions were counted with Quickscint 402 (Zinsser, Frankfurt, F.R.G.) in a $\beta$-counter and corrected for quench-effects after an analogous h.p.l.c. run of [$^{14}$C]AAP alone. Phosphate-content in h.p.l.c.-fractions and incubations was determined after sample preparation according to [17] and measured as described in [18].

## RESULTS

With phospholipid extracts from non-induced mouse liver microsomes incubated with labelled AAP, no radioactivity could be assigned to any lipid peak in an h.p.l.c.-chromatogram when 0.01–0.2 mg extracted phospholipid were applied to a h.p.l.c. column. Therefore we first activated AAP by the HRP/$H_2O_2$ system and investigated the binding of this metabolite to microsomal protein as well as microsomal lipid. Table 1 gives an account of the quantitative relations of the binding of AAP metabolites to microsomal lipid was well as protein. The data show that in livers from untreated animals, no significant lipid binding could be determined. The experiment demonstrates moreover that following methylcholanthrene induction, a small but significant amount of radioactivity was bound to lipid. Finally, when AAP was activated by an artificial model system, the production of reactive metabolite was fast enough to allow detection of lipid as well as protein binding of radioactivity derived from AAP. In any instance, the amount of protein binding was considerably higher than the lipid binding. It was obvious that a reasonable experimental approach as to the nature of lipids affected was only possible with the HRP-system. The experiment shown in Fig. 1 demonstrates that significant amounts of AAP metabolites generated via the HRP reaction were bound to a fraction eluting between the PE and PC fractions. No lipid-bound radioactivity was found under these conditions in the absence of HRP. We recorded also the protein absorption during the h.p.l.c. runs by monitoring the absorbance at 280 nm. No significant signal was observed. In order to corroborate the absence of extracted and possibly labelled lipoprotein, the protein content of the lipid extracts was determined by a much more sensitive method than u.v. absorption. The results showed

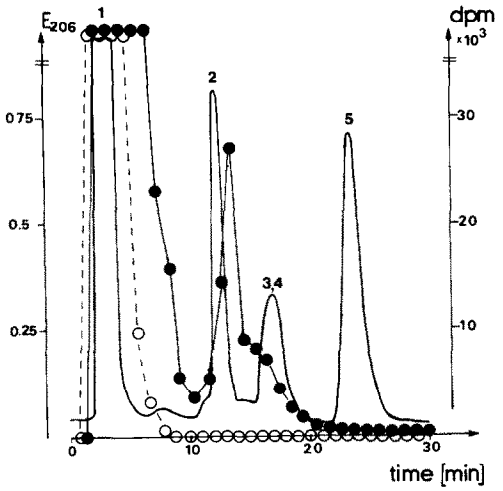


Fig. 1. H.p.l.c.-elution profile of extracted microsomal mouse liver lipids as detected by u.v. absorption (solid line without symbols). Full circles show radioactivity resulting from 10 min incubations at 25° with 1 mM [$^{14}$C]acetaminophen (10 mCi/mole) in the presence of horseradish peroxidase plus $H_2O_2$ generating system. Open circles show radioactivity in the absence of HRP. 0.38 mg extracted phospholipid were applied to a LiChrospher Si 100 column and eluted with a gradient system (see Materials and Methods). 1, neutral lipids and unbound AAP, 2, PE; 3,4, PI and PS; 5, PC.

that the lipid extracts applied to the h.p.l.c. column (with a maximum PL content of 0.5 mg) contained less than 0.5 μg of protein.

Then we returned to the investigation of monooxygenase-generated effects of AAP. In order to improve the yield in bound AAP radioactivity at the given specific radioactivity, for the following experiments microsomes were concentrated after the incubation step. An initial control experiment did not reveal any differences in the elution pattern of phospholipid that had been incubated in the presence of AAP with either intact or boiled microsomes (not shown). Figure 2 shows an elution diagram of phospholipid extracts from AAP-metabolizing mouse liver microsomes. The fractions within the PE as well PC elution region in the experiments with native (vs heat inactivated) microsomes exhibited consistently higher radioactivity. Integrated over all PL fractions, the lipid binding was 0.24 nmol/mg observed with the metabolizing microsomal system. 0.15 nmol/mg lipid binding were found with intact microsomes lacking the NADPH regenerating system. An analogous 'control' binding was observed with the protein binding assay. This means quantitatively that the total amount of lipid binding of AAP caused by enzymatically formed metabolites ranged around $10^{-10}$ mol AAP per mg phospholipid.

We were interested to learn whether extensive metabolic activation of AAP in the presence of pure PE or PC suspensions would result in changes of their elution pattern or labelling. The chromatograms in Figs. 3A and B illustrate that no gross changes in the

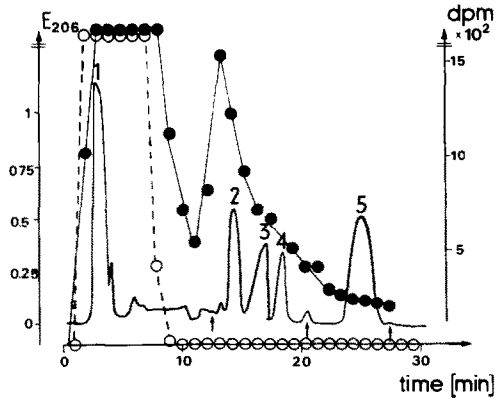


Fig. 2. H.p.l.c.-elution profile of lipids extracted from mouse liver microsomes which had been incubated for 30 min at 37° with 0.8 mM [$^{14}$C]AAP (10 mCi/mmole) in the presence of NADPH. u.v.-absorption is only shown for active microsomes. Full circles show radioactivity profile with active microsomes, open circles with inactivated microsomes. 0.7 mg extracted phospholipid were applied. 1, neutral lipids and unbound AAP; 2, PE; 3, PI; 4, PS; 5, PC; ↑, gradient-derived signals.

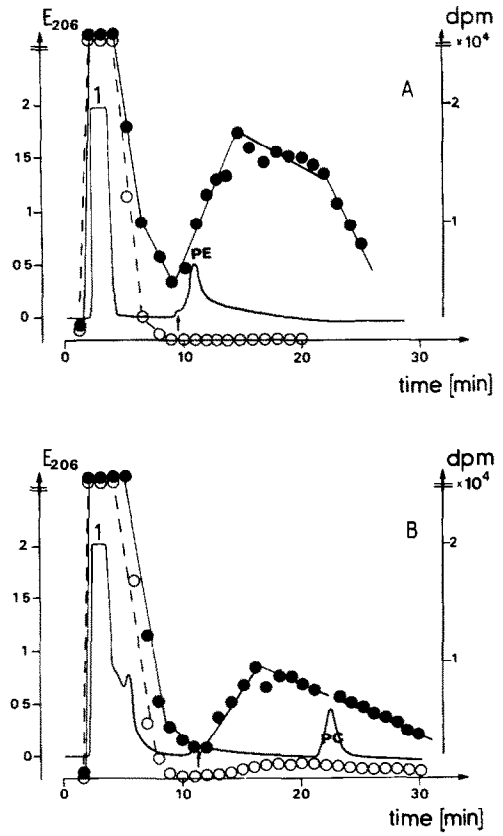


Fig. 3. H.p.l.c.-elution profiles of individual phospholipids which had been incubated with a 1 mM acetaminophen-activating horseradish-peroxidase/glucose oxidase system (full circles) or in the absence of HRP (open symbols). In experiment A, 0.18 mg PE and in experiment B, 0.25 mg PC were applied to the column after an incubation period of 10 min at 25°. The chromatographic system described in Fig. 1. 1, start peak and unbound AAP; 2, PE (A) or PC (B); ↑, gradient-derived signals.

retention times of PE or PC were observed after this treatment. The radioactivity peaks obtained after elution of PE as well as PC incubates were broader than the elution curves of the lipids and essentially not distinguishable from each other. This means that a selective modification of a certain phospholipid class is not possible with this experimental design and was probably not to be expected due to the presence of different fatty acids within single classes.

## DISCUSSION

The experimental aim of this study was to quantitatively demonstrate the binding of AAP metabolites to lipid. To this end, the maximum amount of binding to be expected had to be evaluated first in a model system. The HRP/$H_2O_2$ reaction had been used successfully by others in order to generate a reactive metabolite in a similar way as via the microsomal activation of AAP [19]. An independent methodological necessity was to modify the analytical system [20] in such a way that a maximum separation of unbound AAP from potential target lipids could be achieved. Since essentially no protein was detectable in the lipid extracts of the model or the intact microsomal system, the presence of (lipo)protein-bound radioactivity is likely to be excluded. Moreover, no lipid peroxidation was observed in analogous incubations in the presence of AAP. This is in agreement with our previous observation, that AAP acts *in vitro* in microsomal suspension rather as an inhibitor than as a promotor of lipid peroxidation [11].

An assignment of the binding of AAP metabolites to any individual phospholipid type seems not unequivocally possible for the following reasons. (a) Monooxygenase-generated reactive species are produced within the membrane in a defined molecular lipid environment, whereas HRP-activated metabolites meet microsomal surface lipids first. (b) Although labelled lipid fractions co-elute with PE in the microsomal (Fig. 2) as well as in the HRP system (Fig. 1), experiments with pure PE or PC in suspension (Fig. 3) demonstrate that the labelling pattern does not depend on the type of lipid present. (c) In the absence of an $NADPH_2$-regenerating system a considerable amount of 'control' lipid (0.15 nmol/mg PL) as well as protein (1.2 nmol/mg protein) binding was still detected. In contrast, in the model system lacking HRP or glucose oxidase for $H_2O_2$ generation, essentially no such binding occurred. (d) Recent *in-vivo* work showed that binding of AAP metabolites to lipid is not necessarily limited to carbon–carbon bound, i.e. phospholipids modified at the phosphate group have also been detected [21]. (e) It cannot be excluded that in the HRP/$H_2O_2$ model system some phospholipids were oxidized in such a way [22] that their absorbance (Fig. 1 vs Fig. 2) as well as elution characteristics were substantially changed. The considerations above imply that the central conclusion of this study needs to be limited to the mere demonstration of a lipid binding of microsomal AAP metabolites. If not focused on an individual PL, however, a quantitative estimate seems possible: 0.12 nmol AAP were bound to 0.1 mmol phospholipid in the microsomal assay, i.e. about 0.01% of the phospholipids were chemically modified. This amount is about one order of magnitude less than the molecules which are calculated to be peroxidized following extensive AAP metabolism *in vivo*.

For the primary lesion of paracetamol-induced liver necrosis, at least two independent mechanisms were evoked: the covalent binding and modification of essential proteins [5] and other macromolecules on the one hand, and the peroxidation of polyunsaturated fatty acids on the other hand [9]. A dose-dependence for both processes was demonstrated [10]. Great experimental efforts are needed to demonstrate a presumably covalent lipid modification at all. The functional impairment of membrane dynamics may be aggravated by this process in addition to lipid peroxidation, especially if not abundant but important lipid fractions are affected. Recently, a selective peroxidative modification of phosphatidyl-serine by $CCl_4$-metabolizing microsomes was demonstrated [22]. The authors conclude from the early decrease of the relative abundance of individual fatty acids that this model hepatotoxin induces topologically restricted membrane alterations followed by a defined injury sequence [23]. Interestingly, a group of tri-chloromethylated fatty acids as well as cross-linked acyl chains were very recently identified as the *in-vivo* and *in-vitro* products of aerobic $CCL_4$ metabolism of rat liver [24].

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