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DRUG-INDUCED LIPID PEROXIDATION IN MICE—IV

IN VITRO HYDROCARBON EVOLUTION, REDUCTION OF OXYGEN AND COVALENT BINDING OF ACETAMINOPHEN

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Abstract—A method is described to measure *in vitro* lipid peroxidation by the hydrocarbon evolution technique in cell homogenates or subcellular suspensions of mouse liver. The drug acetaminophen (paracetamol) was shown to quench Fe/ADP-induced as well as NADPH-induced microsomal lipid peroxidation. The pH dependence of ethoxycoumarin deethylation, NADPH–cytochrome P-450 or NADH–cytochrome b_5 reductase activities, and of NADPH-induced microsomal H_2O_2 formation in the presence or absence of acetaminophen was investigated. The presence of the drug at 1 mmole/l caused a shift of the NADPH- or the (NADPH + NADH)-supported H_2O_2 formation to a more alkaline pH. Both processes were inhibited by monooxygenase inhibitors. The dependence of microsomal parameters on the NADP/NADPH ratio was studied and related to the profile of the superoxide dismutase sensitive adrenochrome formation or H_2O_2 formation in the presence and absence of acetaminophen. With a maximum at an NADP/NADPH ratio of 100 a microsomal H_2O_2 production of 5 nmoles/mg protein/min without a concomitant adrenochrome formation was observed only in the presence of acetaminophen. At a NADP/NADPH ratio of 0.01, 8 nmoles/mg/min H_2O_2 and 16 nmoles/mg/min adrenochrome were formed in the absence of acetaminophen. In the presence of 1 mmole/l of the drug, this was quenched to 7 nmoles/mg/min H_2O_2 and 13.5 nmoles/mg/min adrenochrome, respectively. The steady state of the oxy-cytochrome P-450 complex paralleled H_2O_2 formation in the absence of acetaminophen, while it did not so in the presence of it. In further experiments the isocitrate dehydrogenase activity was used to modulate the pyridine nucleotide redox potential: H_2O_2 formation as well as ethane evolution was dissociated into two phases where acetaminophen acted antioxidatively if the pyridine nucleotide couple was reduced. The *in vitro* covalent binding of the drug paralleled H_2O_2 formation as well as ethane evolution. These observations seem to be of general importance with respect to oppositely directed effects of drugs *in vitro* and *in vivo*. Possible mechanisms relating H_2O_2 production, ethane evolution and protein binding to a common radical intermediate are discussed.

The analgesic and antipyretic drug acetaminophen (AAP)† [paracetamol (4-hydroxyacetanilide)] produces severe liver necrosis in animals and man when highly dosed. Morphologically the centrolobular region of the liver is specially affected [1] which is known to be endowed with a higher cytochrome P-450 level [2] and a lower glutathione content [3] than cells within the midzonal part or the periportal zone of the liver lobule. The drug has been frequently used as a model compound for studying mechanisms of toxicity in experimental animals. It has been demonstrated that AAP toxicity is due to the metabolic activation of the parent compound to a reactive intermediate. However, its nature is still under debate. On one hand, detailed *in vivo* and *in vitro* studies of the covalent binding of the radiolabeled drug implicated the inactivation of essential cellular proteins and other macromolecules in mediating the toxic effect (reviewed in Refs 1 and 4). On the other hand, the co-production of reactive oxygen species during drug metabolism *via* the cytochrome P-450 mixed-function oxidase system has been proposed to lead to peroxidation of unsaturated membrane lipids and thus to loss of cell integrity and viability [5–7]. With both experimental approaches similar dose–effect relations were obtained and both views assign a protective role of high specificity to the intracellular thiol, glutathione [6, 8].

In order to test both concepts for a possible common mechanism we studied LPO on a subcellular level by the hydrocarbon evolution technique, as well as *in vitro* protein binding of APP metabolites. Here we report that, in contrast to the *in vivo* situation in mice [7, 9] and to experiments with isolated perfused mouse liver [10], addition of acetaminophen to mouse liver microsomes prevented or decreased LPO.

METHODS

Male albino mice were raised for at least 3 weeks on a commercial semi-synthetic standard laboratory diet [C 1000 (Altromin, Lage, F.R.G.)]. Liver microsomes were prepared according to Ref. 11 in 0.1 mmoles/l potassium phosphate buffer (pH 7.4) containing 1 mmole/l ethylenediaminetetraacetate (EDTA). Microsomes were washed twice in 0.1 moles/l potassium phosphate buffer (pH 7.4) (without EDTA) and finally resuspended in the same

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† Abbreviations: LPO, lipid peroxidation; AAP, acetaminophen (paracetamol).

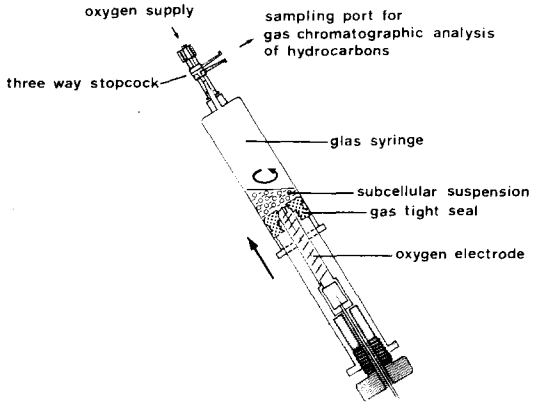

Fig. 1. Measurement of hydrocarbon evolution from tissue homogenates or subcellular fractions *in vitro*.

medium. All assays were, if not stated otherwise, run in 0.1 moles/l phosphate buffer (pH 7.4) at room temp (22°) in duplicate. *In vitro* hydrocarbon evolution was measured while shaking 2 ml of microsomal suspensions at 5 Hz under aerobic conditions. After different times, 7 ml of head space gas were isobarically withdrawn as illustrated in Fig. 1 and analyzed by gas chromatography as described in Ref. 12.

Microsomal enzymes activities were measured as follows: cytochrome P-450 ($\Delta\varepsilon_{450-490} = 91\ cm^{-1} \cdot mM^{-1}$) and cytochrome b_5 ($\Delta\varepsilon_{424-409} = 185\ cm^{-1} \cdot mM^{-1}$) according to Omura and Sato [13] on a Shimadzu UV-300 dual-wavelength spectrophotometer. The oxy-P-450 steady state was determined as $\Delta A_{442-470}$ [14]. Further microsomal parameters included determination of ethoxycoumarin deethylation [15], *N*-demethylation of aminopyrine [16] and hydroxylation of aniline [17]. *Superoxide dismutase sensitive* adrenochrome formation was measured according to Ref. 18 using $\Delta\varepsilon_{485-475} = 2.96\ cm^{-1} \cdot mM^{-1}$; blanks were assays in the presence of 10 U superoxide dismutase. H_2O_2 production was determined as described in Ref. 19. This indicator system based on the formaldehyde production from methanol *via* catalase was found to be insensitive to the AAP concns used, in contrast to the horseradish peroxidase system. Furthermore the NADPH-regenerating system was checked for possible interference with the AAP added. Less than 10% activity was lost up to concns of 10 mmoles/l AAP. Reduced pyridine nucleotide–ferricyanide reductase activity, referred to later on as NADPH–cytochrome P-450 reductase activity, was determined as described in Refs 20 and 21 using $\varepsilon_{420} = 1.02\ cm^{-1} \cdot mM^{-1}$. Essentially the same results were obtained when ferricytochrome *c* [20, 21] was used as the electron acceptor.

Redox titrations were carried out in the presence of initially 50 μM reduced pyridine nucleotide with increasing amounts of $NAD(P)^+$. Redox potentials were calculated on the basis of a standard redox potential for the pyridine nucleotide redox couple of $E_0' = -317$ mV, corrected for pH 7.4. Each point represents an independent experiment and the enzymatic activities are given as initial rates.

Protein binding was studied by incubating 1 mmole/l [^{14}C]AAP (Amersham, sp. act. 0.5 μCi/μmole, radiochemical purity >99%, checked by high-performance liquid chromatography in the system of Ref. 22) with 1 mg/ml microsomal protein for 30 min at 22° in 0.1 moles/l potassium phosphate (pH 7.4). Fifty microlitres were transferred to a cellulose filter disk (Whatman, 30 mm dia.), immersed in 15 g/100 ml trichloroacetic acid, then extracted as described in Ref. 23. Chemicals used were: 7-ethoxycoumarin, acetaminophen, L-epinephrine, DL-isocitrate, rotenone and superoxide dismutase from Sigma (München, F.R.G.); aminopyrine was from Ega-Chemie (Steinheim, F.R.G.), sodium diethyldithiocarbamate from Fluka (Buchs, Switzerland); metyrapone from Ciba-Geigy (Basel, Switzerland); pyridine nucleotides and catalase (65 kU/mg protein) from Boehringer (Mannheim, F.R.G.). SKF 525 A (2-diethylaminoethyl-2-diphenylpropylacetate) was a gift from Smith, Klinefeld & French (Bruxelles, Belgium).

RESULTS

Mouse liver homogenate was incubated at a protein concn of 25 mg/ml with the addition of 5 mmoles/l of various compounds in the presence of an NADPH-regenerating system and ethane evolution was monitored over a 1-hr period. Among several agents, including octylamine, phenetidine, cyclophosphamide, isoniacide, acetanilide, phenacetin and *p*-nitroanisol, acetaminophen was found to inhibit LPO in these liver homogenates. Further experiments were carried out with mouse liver microsomes. Their biochemical characteristics are given in Table 1 and are comparable to the data reported for the rat [23]. Figure 2 shows that the inhibitory action of acetaminophen upon NADPH-induced microsomal LPO is concn-dependent and

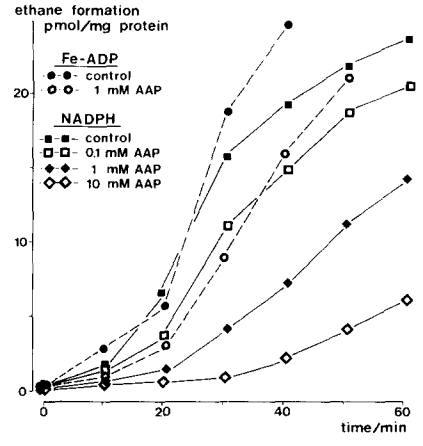

Fig. 2. *In vitro* lipid peroxidation of mouse liver microsomes induced by 0.1 mmoles/l $FeCl_3$, 2 mmoles/l ADP plus 1 mmole/l ascorbate (circles) or by the presence of NADPH (including regenerating system) and its quenching by acetaminophen (squares). Conditions: 0.1 mmoles/l potassium phosphate (pH 7.4); 2 mg/ml protein; 22°; 3.3 mmoles/l $MgCl_2$; 1 mmole/l $NADP^+$; 10 mmoles/l isocitrate; 0.5 U/ml isocitrate dehydrogenase; 1 μg/ml rotenone; total vol. 1 ml.

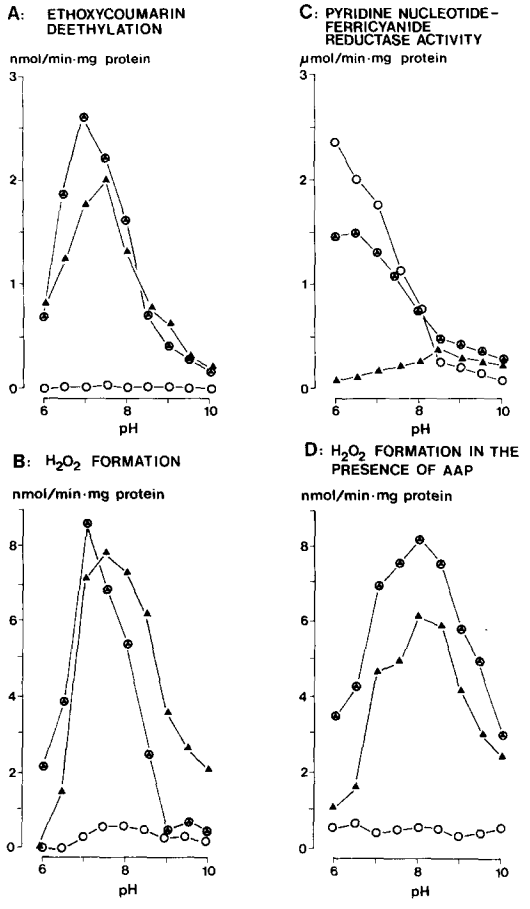

Fig. 3. pH profile of mouse liver microsomal enzyme activities as well as of microsomal H_2O_2 formation rates in the presence of NADH (○), NADPH (▲) or NADH + NADPH (◭). Conditions: as indicated in Fig. 2, except: 10 mmoles/l Tris–HCl; 10 mmoles/l Bi-tris, 10 mM borate buffers of varying pH; 0.1 mmoles/l pyridine nucleotide(s); 1 mmole/l acetaminophen.

is also observed when LPO is initiated by Fe/ADP in microsomal suspensions. Several control experiments were done in order to rule out a possible blocking by AAP of microsomal electron flow at different sites. NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase (both assayed by cytochrome *c* reduction) were not sensitive to additions of AAP up to 1 mmole/l. At 10 mmoles/l (inhibitions of 15 and 10%, respectively, were obtained. Ethoxycoumarin dealkylation rates were similarly independent from AAP added up to 1 mmole/l, while at 10 mmoles/l a 35% competitive inhibition was observed. Thus, with an intact microsomal electron transfer, acetaminophen acts *in vitro* as an antioxidant, in contrast to the *in vivo* situation.

In order to further characterize this antioxidant function, the pH dependence of microsomal enzymes was studied in comparison with the pH profile of a prooxidant microsomal process, i.e. H_2O_2 formation. Figure 3A shows the expected activity optimum of the NADPH-supported ethoxycoumarin dealkylation at pH 7.5, the lack of cosubstrate function of NADH and the substrate synergism of NADPH plus NADH accompanied by a shift of the optimum to

Table 1. Biochemical characterisation of mouse liver microsomes obtained from five individual animals

Cytochrome content (nmoles/mg protein)	
P-450	0.72 ± 0.04
b_5	0.65 ± 0.12
Sp. acts (nmoles/mg protein/min)	
Aniline hydroxylation	0.39 ± 0.01
Aminopyrine *N*-demethylation	1.53 ± 0.55
Ethoxycoumarin *O*-deethylation	1.77 ± 0.62
NADPH–ferricyanide reductase	268 ± 35
NADH–ferricyanide reductase	2493 ± 110

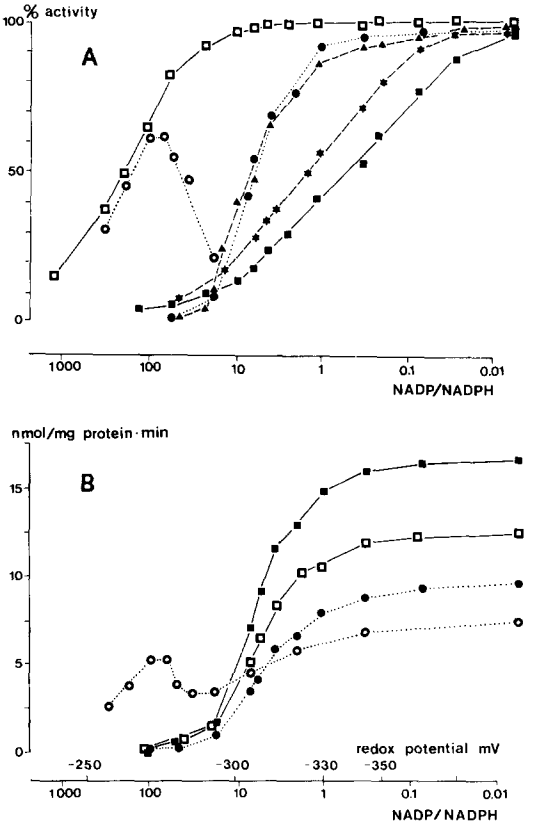

Fig. 4. Influence of the pyridine nucleotide redox state on microsomal enzyme activities (A) and on the formation of reactive oxygen species (B) (conditions as in Fig. 2, pH = 7.4). (○—○), AAP-induced H_2O_2 formation [data from B *minus* spontaneous H_2O_2 formation (%)]; (●—●), spontaneous H_2O_2 formation [data from B (%)]; (▲—▲), steady-state oxy-P-450 ($\Delta A_{442-470}$); (★—★), ethoxycoumarin deethylase; (■—■), NADPH–ferricyanide reductase; (□—□), NADH–ferricyanide reductase (NAD$^+$/NADH as variable!). Table 1 contains the 100% values for the enzymatic activities (steady-state oxy-P-450: 100% corresponding to 7×10^{-3} O.D./mg protein). (B) (■—■), Spontaneous (NADPH-induced) superoxide dismutase sensitive adrenochrome formation; (□—□), adrenochrome formation in the presence of 1 mmole/l AAP; (●—●), spontaneous (NADPH-induced) H_2O_2 formation; (○—○), H_2O_2 formation in the presence of 1 mmole/l AAP.

Table 2. Influence of monooxygenase inhibitors on H_2O_2 formation (nmoles/mg microsomal protein/min) in mouse liver microsomes

Additions	−AAP		+1 mmole/l AAP	
	pH = 7.5	pH = 8	pH = 7.5	pH = 8
NADPH (0.1 mmoles/l)	6.9	7.8	4.9	8.0
NADPH + SKF 525 A	3.4	3.0	2.1	1.9
NADPH + metyrapone	1.5	3.4	2.9	0.7
NADPH + naphthoflavone	8.9	8.4	5.2	8.2
NADPH + dithiocarbamate	4.7	6.1	3.4	5.4

Conditions: 250 μmoles/l inhibitor, except naphthoflavone (100 μmoles/l); 2 mg/ml microsomal protein; 22°.

pH 6.9. Comparison of Fig. 3A and B demonstrates that pyridine nucleotide induced H_2O_2 formation (i.e. the spontaneous H_2O_2 formation in the absence of exogenous substrate) and ethoxycoumarin dealkylation pH profiles are very similar. This confirms the recently suggested view [24, 25] that both products are derived from the same reaction, i.e. an oxy-P-450 intermediate. In contrast, microsomal H_2O_2 formation in the presence of AAP is maximal at pH 8.1 with NADPH as well as with both reduced pyridine nucleotides as substrates. This finding suggests the microsomal AAP-induced H_2O_2 formation is a process different from the pyridine nucleotide induced spontaneous H_2O_2 production. Nevertheless, cytochrome P-450 dependent partial reactions seem to be involved, since both types of H_2O_2 formation are inhibited by common monooxygenase inhibitors (Table 2). With respect to inhibition, no different pattern was elaborated between pH 7.4 and 8 suggesting that P-450 activity is needed for both processes.

It appeared that an analogy with the pH dependence is not sufficient to pinpoint the source of AAP-induced H_2O_2 formation. Therefore new experiments were carried out where the NADP/NADPH ratio was used as a variable. The data in Fig. 4A demonstrate a similar dependence of ethoxycoumarin deethylase and NADPH–cytochrome-P-450 reductase activity from the pyridine nucleotide redox state, and a very close correlation between the oxy-cytochrome P-450 steady state and the spontaneous H_2O_2 production, suggesting again that this H_2O_2 arises from the decay of the oxy-complex. The same type of dependence from the NADP/NADPH ratio is seen with the adrenochrome level (Fig. 4B, upper curve) supporting the interpretation that this H_2O_2 may be formed *via* spontaneous dismutation of superoxide. The constant 2 to 1 stoichiometry of adrenochrome to H_2O_2 over four decades of the pyridine nucleotide ratio provides additional strong support for this view. In the presence of AAP, however, different conditions apply: below about −310 mV AAP quenches H_2O_2 as well as adrenochrome formation. Above this redox potential, AAP-induced H_2O_2 formation is enhanced with a maximum around −270 mV, while the decrease of spontaneous H_2O_2 as well as both types of adrenochrome formation with increasing redox potential seem unaffected by the presence of AAP. This substantiates the conclusion drawn from the pH dependence experiment, i.e. that microsomal AAP-induced H_2O_2 formation is a separate process, obviously not accompanied by a superoxide dismutase sensitive adrenochrome formation. In total, the experiment demonstrates that the initial pyridine nucleotide redox state is a parameter that decides whether AAP acts *in vitro* as a stimulator or a quencher of microsomal H_2O_2 production.

In order to further test this hypothesis, we studied the dependence of H_2O_2 formation, ethane evolution and protein binding caused by AAP on the rate of pyridine nucleotide regeneration by varying the amount of isocitrate dehydrogenase in the assay Fig. 5. The redox ratio of the NADP/NADPH pool in the presence of isocitrate dehydrogenase that led to the peroxidative response was about the same as in the previous experiment (see Fig. 4). Furthermore the rate of H_2O_2 formation as well as the extent of inhibition by AAP under low NADP/NADPH conditions are quantitatively similar in both experiments. In agreement with the preceding experiment a decrease in isocitrate dehydrogenase activity from 0.5 U (as used in the standard incubation) to zero continues to decrease the spontaneous H_2O_2 production as well as ethane evolution. Evidently, the presence of AAP at a high NADPH-regenerating capacity quenches both prooxidative processes. Below a cross-over point around 30–40 mU of isocitrate dehydrogenase in the assay, the opposite effect of AAP is observed, i.e. AAP enhances H_2O_2 as well as ethane formation. The difference between AAP-induced and spontaneous H_2O_2 formation again should be due to a separate process. The point, however, is that the protein binding of AAP shows the same characteristics as H_2O_2 and ethane formation suggesting a common origin of the three events.

DISCUSSION

Methodological contribution

The determination of *in vitro* formed hydrocarbons in order to assess LPO has been described in systems where rat liver microsomes [26] or parenchymal cells [27, 28] were incubated in head space vessels or in Erlenmeyer flasks. Here we describe a technically simple system which allows us to study alkane formation in a 1-ml suspension, to remove gas several times without changes in pressure or distribution equilibria, and to control aerobiosis of the incubate. With the gas chromatographic system used [12] 1 pmole alkane formed from 1 ml of a

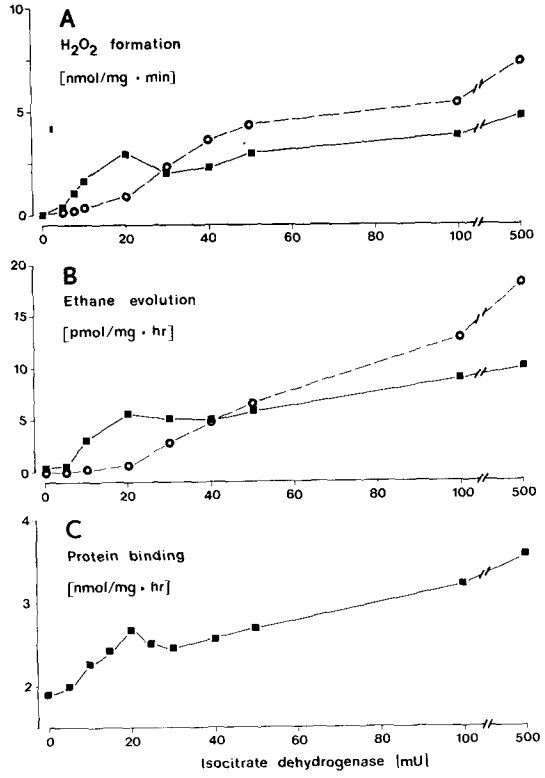

Fig. 5. Dependence of microsomal spontaneous (circles) or AAP-induced (squares) H_2O_2 formation and ethane evolution, as well as protein binding of [^{14}C]AAP or the activity of the NADPH-regenerating system in the assay. Conditions: as in Fig. 2, except A and B (2 mg/ml protein); (A) 50 mmoles/l methanol + 5000 U/ml catalase; (C) 1 mg/ml protein; controls: isocitrate dehydrogenase as indicated, but in the absence of NADP.

suspension containing 1 mg of microsomal protein is reliably determined.

Mechanistic aspects

The idea of a chemical which is trapping a radical by becoming itself a less reactive or metabolically removable radical implicates a general rationale for the *in vitro* effects observed here. The finding that AAP quenches *in vitro* not only the NADPH-supported microsomal LPO but also the Fe/ADP-induced ethane formation indicates that complete turnover of AAP *via* the monooxygenase system is not necessary to evoke this effect. The Fe/ADP experiment suggests rather a redox cycle of AAP where a LPO-related radical withdraws an electron from AAP and a microsomal reductase or ascorbate reduce the oxidized AAP intermediate. This also implies that less reduced pyridine nucleotide would be available for the microsomally catalyzed production of reactive oxygen species. Actually, derived from theoretical considerations [29] as well as on an experimental basis [30], a semiquinone intermediate of AAP has been postulated as a possible reactive microsomal species.

At this point, this discussion has to be extended in order to reconcile the apparently controversial effects of AAP in the intact liver compared to the disintegrated organ. Several possibilities seem conceivable for the ensuing reaction which the primary AAP intermediate may undergo *in vivo*, such as redox cycling, non-enzymatic activation of oxygen, or decay of an intermediate downstream of the oxy-P-450 complex. The scheme in Fig. 6 is aimed to reconcile the complexity of suggestions and findings. In situation A—which is considered to be the relevant one—a cytochrome P-450 dependent H_2O_2 production *via* dismutation of O_2^- could lead to LPO

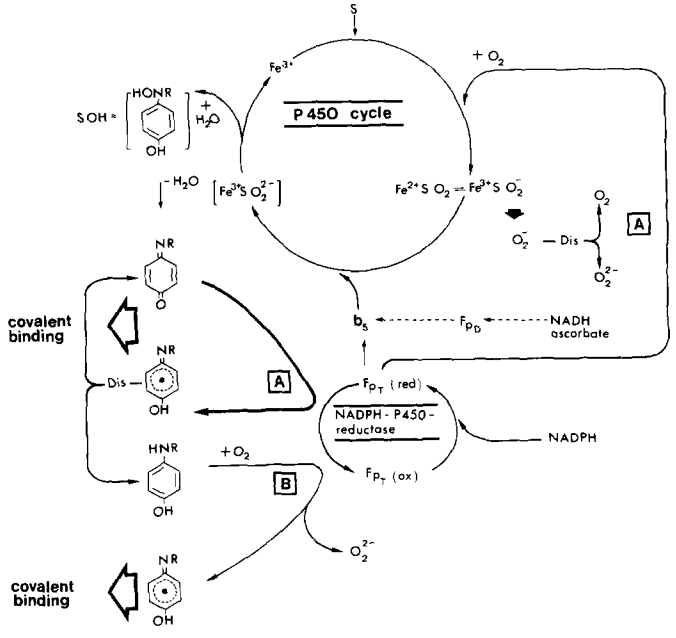

Fig. 6. Proposed mechanisms mediating the *in vitro* quenching effect of acetaminophen on lipid peroxidation and covalent binding at low NADP/NADPH (A) and high NADP/NADPH (B) ratios. s = substrate = acetaminophen, F_{P_D} = NADH–cytochrome b_5 reductase bound flavoprotein; F_{P_T} = NADPH–cytochrome P-450 reductase bound flavoprotein; Dis = dismutation reaction.

in vivo, which is quenched *in vitro* when the putative reaction product of the substrate AAP, either a quinoneimine or a semiquinone, withdraws electrons from the NADPH–cytochrome P-450 reductase.

The data in Figs 4 and 5 need a rationale as to why, under an artificially high NADP/NADPH ratio, the presence of AAP induces a two-electron reduction of oxygen accompanied by formation of an AAP species undergoing covalent binding. Situation B illustrates conjectively that this could happen if the reduced flavoprotein of NADPH–cytochrome P-450 reductase autoxidizes in the simultaneous presence of AAP and O_2. If oxygen takes up one electron from the flavoprotein and another one from AAP, H_2O_2 not involving superoxide as well as a semiquinone are formed. Such a reaction has not yet been described for AAP; however the oxidation of *N*-acetylaminoarenes by ferricyanide [31] may provide a suitable model for this type of reaction. The involvement of oxygen as substrate and product seems to exclude a diaphorase-type reaction [32].

Toxicological implications

The observation that a drug acts *in vivo* as an inducer of LPO but quenches it *in vitro* has serious consequences as to the validity of *in vitro* screening of such compounds. Generally, in risk assessment, the chronological course of the study begins with *in vitro* prescreening bearing in this case the hazard of underestimating the xenobiotic potential of the compound, or of overestimating the potential therapeutic usefulness.

General significance

In vivo [7] as well as in the isolated perfused mouse liver [10] AAP was shown to increase lipid peroxidation in a dose-dependent manner. In isolated hepatocytes on the other hand, two *in vivo* prooxidative drugs, i.e. ethylmorphine and aminopyrine [7], failed to increase LPO assayed by different methods [27]. Here we report that in cell homogenates or microsomal suspensions an "antioxidative" effect of AAP is observed. In the preceding paper [8], from the differential biological potency of free and liposomal GSH in mouse liver *in vivo*, the conclusion was reached that the structural organisation within the organ is of utmost importance for metabolic efficiency. This study provides another example that by passing from the perfused organ to cellular fractions a defined biochemical balance may be turned in the opposite direction or control of it may be lost. This knowledge seems not to be generally translated into practical awareness.

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