Biochemical Pharmacology, Vol. 35, No. 21, pp. 3693–3699, 1986.
Printed in Great Britain.

0006–2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

PARACETAMOL, 3-MONOALKYL- AND 3,5-DIALKYL DERIVATIVES

COMPARISON OF THEIR MICROSOMAL CYTOCHROME P-450 DEPENDENT OXIDATION AND TOXICITY IN FRESHLY ISOLATED HEPATOCYTES

R. VAN DE STRAAT, J. DE VRIES, T. KULKENS, A. J. J. DEBETS and N. P. E. VERMEULEN*

Department of Pharmacochemistry, Molecular Toxicology, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

(*Received* 20 *January* 1986; *accepted* 23 *April* 1986)

Abstract—The effects of 3-monoalkyl- and 3,5-dialkyl-substitution on the cytotoxicity of paracetamol (PAR) in rat hepatocytes was studied. PAR is known to be bioactivated by the hepatic microsomal cytochrome P-450 containing a mixed-function oxidase system presumably to *N*-acetyl-para-benzoquinone imine (NAPQI), a reactive metabolite which upon overdosage of the drug causes depletion of cellular glutathione (GSH) and hepatotoxicity. The four 3-mono- and the four 3,5-di-alkyl-substituted derivatives of PAR investigated in this study (R = CH_3, C_2H_5, C_3H_7, C_4H_9) interacted with cytochrome P-450 giving rise to reverse type I spectral changes. Like PAR, all derivatives underwent cytochrome P-450-mediated oxidation to NAPQIs. In contrast to induction by phenobarbital, induction of cytochrome P-450 by 3-methylcholanthrene enhanced the microsomal oxidation of PAR and its derivatives. The NAPQIs formed from PAR and the 3-mono-alkly derivatives by microsomal oxidation were found to conjugate with GSH and to oxidise GSH to GSSG. The NAPQIs formed from the 3,5-dialkyl-substituted derivatives, however, only oxidized GSH to GSSG. PAR and the 3-monoalkyl derivatives were found to deplete cellular GSH to about the same extent and to be equally toxic in freshly isolated hepatocytes from 3-methylcholanthrene treated rats. In contrast, the 3,5-di-alkyl-substituted derivatives of PAR did not affect the GSH levels and were not toxic in the hepatocytes, even at higher concentrations. It is suggested that the difference between the way of reacting of 3,5-dialkyl-NAPQIs and NAPQIs from PAR and 3-monoalkyl derivatives with thiols of cellular GSH and protein could account for the observed difference between the toxicity of the 3,5-dialkyl- and the 3-monoalkyl-substituted derivatives of PAR.

Paracetamol (acetaminophen, 4-hydroxyacetanilide; PAR†) is a commonly used analgesic drug which after overdosage has been shown to cause centrilobular hepatic necrosis in several species [1, 2]. At normal levels, the drug mainly undergoes glucuronidation and sulfation in the liver. A small part, however, is bioactivated by the hepatic cytochrome P-450-containing MFO system to a toxic reactive metabolite, presumably NAPQI [3, 4]. This electrophilic reactive intermediate is normally detoxified by conjugation to reduced GSH under formation of the 3-glutathionyl conjugate. Upon overdosage of PAR, however, the glucuronidation and sulfation routes are saturated and extensive oxidative bioactivation of PAR to NAPQI occurs, resulting in a depletion of cellular GSH pools. Subsequent covalent binding of the reactive intermediate to thiol groups of cysteine residues in proteins [5] is generally thought to be involved in the initiation of cell toxicity [6, 7], although, more recently, the participation of an oxidative stress has been suggested as a possible alternative accounting for the hepatocellular toxicity of PAR [8–10].

The bioactivation of PAR is cytochrome P-450 isoenzyme selective, i.e. the cytochrome P-450 isoenzymes induced by 3-MC are highly active in the bioactivation of PAR, whereas the PB-inducible isoenzymes are inactive [11, 12]. Presumably, therefore, PAR is more hepatotoxic in 3-MC pretreated animals when compared to control or PB pretreated animals [13, 14].

In principle, an approach to design a safer drug is to modify, on the basis of insights into the molecular mechanism of toxicity, its chemical structure in such a way that the adverse activities are diminished without lowering of the therapeutic activity. In the case of PAR, it has been shown that mono- and di-alkyl-substitution at the 3 and 5 positions of the aromatic nucleus did not reduce the analgesic activity [15, 16]. Such kinds of substitutions might affect the hepatotoxicity of the drug, on the level of interaction with cytochrome P-450 and oxidation by the cytochrome P-450 containing MFO system by a change in physico-chemical properties such as lipophilicity, or more directly by blocking the reactive positions of NAPQI, the presumed toxic intermediate.

* To whom correspondence should be addressed.

† Abbreviations: PAR, paracetamol; MFO, mixed-function oxidase; NAPQI, *N*-acetyl-para-benzoquinone imine; GSH, reduced glutathione; GSSG, oxidized glutathione; 3-MC, 3-methylcholanthrene; PB, phenobarbital; LDH, lactate dehydrogenase; DMSO, dimethylsulfoxide; DTPA, diethylenetriamine pentaacetic acid.

Table 1. Structure and lipophilicity of PAR and the 3-monoalkyl- and 3,5-dialkyl-substituted derivatives

Compounds		R_1	R_2	Log P*
I	PAR	H	H	0.311
II	3-methyl-PAR	CH_3	H	0.793
III	3-ethyl-PAR	C_2H_5	H	1.306
IV	3-isopropryl-PAR	C_3H_7	H	1.707
V	3-t-butyl-PAR	C_4H_9	H	2.357
VI	3,5-dimethyl-PAR	CH_3	CH_3	1.108
VII	3,5-diethyl-PAR	C_2H_5	C_2H_5	1.870
VIII	3,5-diisopropyl-PAR	C_3H_7	C_3H_7	2.671
IX	3,5-di-t-butyl-PAR	C_4H_9	C_4H_9	3.180

R_1 and R_2 are substituents at the 3- or at the 5-position in the aromatic nucleus of 3-hydroxyacetanilide.
* Taken from ref. 17.

The aim of the present study was to investigate the effects of 3-monoalkyl- and 3,5-dialkyl-substitution on the binding of PAR to cytochrome P-450, its cytochrome P-450-containing MFO-mediated oxidation as well as its cytotoxicity in freshly isolated hepatocytes.

MATERIALS AND METHODS

Chemicals. PAR and the 3-monoalkyl- and 3,5-dialkyl-substituted derivatives investigated are listed in Table 1. The compounds were synthesized from their corresponding phenols as described by Dearden and O'Hara [17]. 3-t-Butyl-PAR (V) and 3,5-di-t-butyl-PAR (IX) were a generous gift of Dr J. C. Dearden.

PB and PAR were obtained from Brocacef (Delft, The Netherlands). Collagenase, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, GSH, GSSG, catalase, superoxide dismutase, pyruvate, lactic acid, NADPH and NADH were obtained from Boehringer (Mannheim, F.R.G.). Imidazole, DTNB, 3-MC, DTPA, *N*-ethylmaleimide, *o*-phthalic-dicarboxaldehyde and substituted phenols were purchased from Janssen (Beerse, Belgium). Potassium phosphate, $MgCl_2$, DMSO, trichloroacetic acid, acetic acid and methanol came from Baker (Deventer, The Netherlands). Bovine serum albumin and γ-glutamiltranspeptidase were obtained from Sigma (St. Louis, MO). SKF-525A was a gift from Smith, Kline & French (Brussels, Belgium). Ellipticine was a gift from the National Cancer Institute (U.S.A.).

Animals, preparation of microsomes and isolation of freshly isolated hepatocytes. Male albino Wistar rats (180–200 g) were used. In the case of pretreatment of animals, PB was administered orally during 7 days (1 mg/ml drinking water) and 3-MC (40 mg/kg, dissolved in arachides oil) was injected once intraperitoneally 48 hr before use. Rats were fasted overnight before use. Microsomes were prepared by difference ultra centrifugation [18] and stored at −180° at a concentration of 15 mg protein per ml; protein was determined by the method of Bradford [19]. Hepatocytes were isolated by collagenase perfusion essentially according to the procedure described by Seglen [20]. Modifications of the isolation procedure were as follows: Krebs–Henseleit bicarbonate buffers (pH 7.4) saturated with 95% oxygen and 5% carbon dioxide were used throughout the isolation of hepatocytes. The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The livers were perfused with calcium free buffer, firstly through the vena porta after opening of the lower vena cava and subsequently through the upper part of the vena cava after closing of the lower part of the vena cava. After several washing steps the cells were suspended in cold buffer containing 2% (w/v) bovine serum albumin. Cells isolated by this method usually contained over 90% viable cells as judged by trypan blue exclusion and LDH leakage.

Difference spectroscopy. Spectral studies on substrate binding to hepatic microsomal cytochrome P-450 were performed with an Aminco DW-2 spectrometer, as described by Jefcoate [21]. Samples (3 ml) comprised 3 mg of microsomal protein in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM DTPA. PAR and the 3-monoalkyl- and 3,5-dialkyl derivatives were added to the sample cuvette dissolved in DMSO; equal amounts of DMSO were added to the reference cuvette. Maximally 2 μl of DMSO were added. K_s values were calculated from double reciprocal Lineweaver–Burke plots of the difference in absorption at 420 and 390 nm and the substrate concentrations.

Microsomal incubations. Incubation mixtures (1 ml) consisted of 5 mM glucose-6-phosphate, 5 mM $MgCl_2$, 0.04 IU glucose-6-phosphate dehydrogenase, 1 mM GSH, 2000 U catalase, microsomes (1 mg) and 1 mM of the substrates in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM DTPA. After preincubation for 5 min at 37°, the reaction was initiated by addition of NADPH (1 mM). After 10 min, the reaction was terminated by addition of 0.5 ml 25% (w/v) cold trichloroacetic acid. The formation of GSSG was determined as described by Hissin and Hilf [22]. Catalase was added to the incubation mixture to prevent oxidation of GSH by active oxygen species during incubation, additional superoxide dismutase had no effect. GSH-conjugates formed were determined by HPLC [23]. In the case of PAR, 3-methyl-PAR (II), 3-ethyl-PAR (III), 3-isopropyl-PAR (IV) and 3-t-butyl-PAR (V) the mobile phase consisted of 18, 27, 36, 45 and 54% (v/v) methanol in 1% (v/v) acetic acid in H_2O, respectively. The GSH-conjugates were identified by comparison of their retention times with those of synthetic standards and by breakdown by γ-glutamyltranspeptidase [24]. The effect of nitrogen was studied by flushing the incubation mixtures with nitrogen during preincubation and incubation. K_m values were calculated from double reciprocal Lineweaver–Burke plots of the rate of GSH oxidation or conjugation and the concentrations of substrates.

Incubation of hepatocytes. Freshly isolated hepatocytes (1.5–2 × 10^6 cells/ml) were incubated in 3 ml vol. of Krebs–Henseleit buffer containing 2% bovine serum albumin, 10 mM lactic acid and 1 mM pyruvate. The cells were equilibrated at 37° with 95% oxygen, 5% carbon dioxide for 15 min, prior to addition of PAR, the 3-monoalkyl- and 3,5-dialkyl derivatives dissolved in incubation medium. During incubation aliquots of 1 ml were taken and centri-

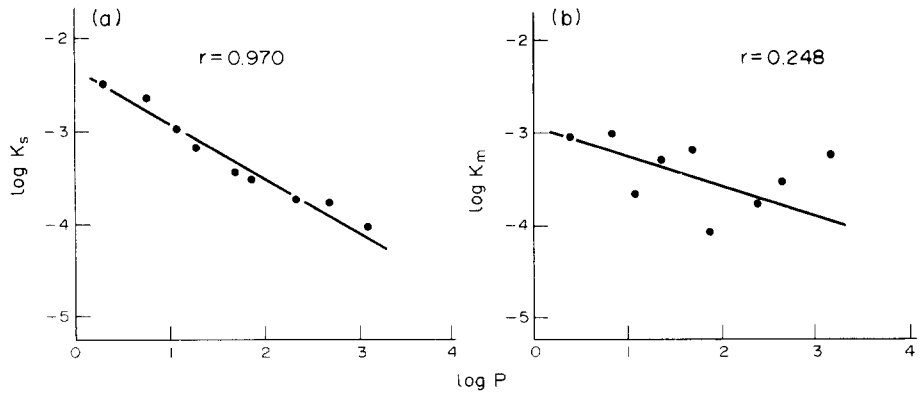

Fig. 1. Relationship between log P and log K_s (A) and log P and log K_m (B) of PAR (I), its 3-monoalkyl- (II,III,IV,V) and its 3,5-dialkyl derivatives (VI,VII,VIII,IX). K_s and K_m were determined as described in Materials and Methods, log P values were taken from ref. 17. Results represent means of two separate experiments.

Table 2. Formation of GSSG from GSH during microsomal incubation of 1 mM 3,5-dimethyl-PAR (VI) in the presence of NADPH and oxygen

Incubation	% GSSG formed
Complete	100
− NADPH	1
− 3,5-dimethyl-PAR	18
+ Nitrogen	39
+ SKF 525a (2.5 mM)	38
+ Imidazole (0.5 mM)	61
+ Ellipticine (0.5 mM)	76

Incubations and GSSG determinations were performed as described in Materials and Methods.

Results are expressed as means of two separate experiments. The absolute value of a complete incubation (100%) was 1.2 ± 0.1 nmol GSSG/mg protein/min.

fuged (60 g for 1 min), 0.8 ml of cell free supernatant was taken for the assay of LDH [25]. LDH activity was expressed as a percentage of the activity present in a homogenized cell suspension. To the pellet 100 μl of 15% (w/v) trichloroacetic acid was added to precipitate protein. The supernatant obtained after 15 min centrifugation at 5000 g was used for GSH determination [26].

RESULTS

Spectral interaction

Upon addition of PAR, the 3-monoalkyl- or 3,5-dialkyl-substituted derivatives to liver microsomes from untreated or from 3-MC- or PB-pretreated rats, difference spectra were observed with an absorption peak at about 419 nm and a trough at about 388 nm.

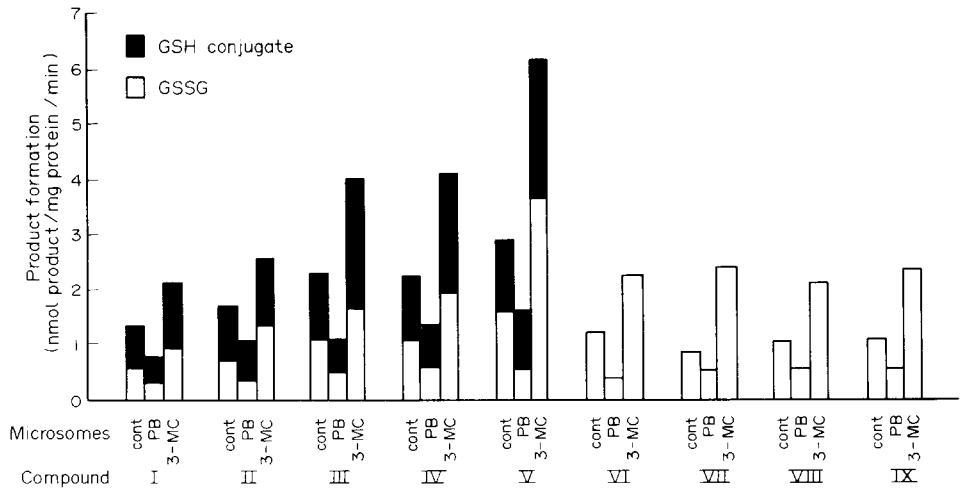

Fig. 2. Formation of GSSG and GSH-conjugates during incubation of PAR (I), its 3-monoalkyl- (II,III,IV,V) and its 3,5-dialkyl derivatives (VI,VII,VIII,IX) with hepatic microsomes from control, PB treated and 3-MC treated rats in the presence of NADPH, GSH and oxygen. Incubations and determinations were performed as described in Materials and Methods. Results represent means of two separate experiments.

Table 3. Cytotoxicities of PAR, 3-methyl-PAR (II) and 3,5-dimethyl-PAR (VI) in freshly isolated hepatocytes from untreated and 3-MC treated rats

Addition	% LDH leakage after 3 hr incubation	
	Control	3-MC treated
None	11 ± 4	15 ± 2
PAR (0.5 mM)	28 ± 6	60 ± 6
3-Methyl-PAR (0.5 mM)	20 ± 7	54 ± 4
3,5-Dimethyl-PAR (0.5 mM)	14 ± 4	17 ± 3

Incubations of hepatocytes and determination of LDH leakage were performed as described in Materials and Methods.

LDH leakage is expressed as a percentage of total activity in cells. Results are expressed as means ± SD of 3 separate experiments in case of untreated rats and as means ± SD of 5 separate experiments in the case of hepatocytes from 3-MC treated rats.

All spectra showed reverse type I spectral changes. Neither PAR nor its derivatives were able to displace haeme binding ligands like imidazole or carbon monoxide from their binding sites, a criterion for discrimination between a type II and a reverse type I spectral change [27]. Both 3-mono- and 3,5-di-alkyl-substitution of PAR decreased the values of K_s with increasing alkyl-substitution. A good correlation ($r = 0.970$) was observed between the K_s determined with microsomes from 3-MC treated rats and the lipophilicity of PAR and all of its alkyl-substituted derivatives (Fig. 1A).

Microsomal oxidation

The possible formation of *N*-acetyl-quinone imine metabolites from PAR and its derivatives by microsomal cytochrome P-450 mediated oxidation was investigated by studying two reactions of synthetic NAPQI with GSH, i.e. oxidation of GSH to GSSG and conjugation to GSH [28].

PAR and its 3-monoalkyl- and 3,5-dialkyl-substituted derivatives gave rise to GSSG formation on incubation with microsomes in the presence of GSH and NADPH. As illustrated for 3,5-dimethyl-PAR (VI) in Table 2, oxidation of GSH was found to be NADPH- and oxygen-dependent, indicating the involvement of the cytochrome P-450 containing MFO system. Furthermore, the cytochrome P-450 inhibitors SKF 525A, imidazole and ellipticine [29] decreased GSSG formation (Table 2).

A discrepancy was found between PAR and the 3-monoalkyl-substituted derivatives on the one hand and the 3,5-dialkyl-substituted derivatives on the other hand in the way of reacting of their metabolites with GSH. The reactive metabolites of PAR and its 3-monoalkyl derivatives conjugated with GSH as well as oxidized GSH. In contrast, the 3,5-dialkyl-substituted derivatives yielded reactive metabolites which only oxidized GSH to GSSG (Fig. 2).

Pretreatment of rats with PB or 3-MC differentially affected the microsomal oxidation of PAR and the 3-monoalkyl- and 3,5-dialkyl-substituted derivatives. As shown in Fig. 2, cytochrome P-450 induction by 3-MC increased the oxidation of all compounds whereas induction by PB decreased it. Product formation was found to obey Michaelis–Menten kinetics. The K_m values of PAR, 3-monoalkyl- and 3,5-dialkyl derivatives obtained upon incubation with hepatic microsomes from 3-MC pretreated rats did not correlate with the lipophilicity of the compounds (Fig. 1B).

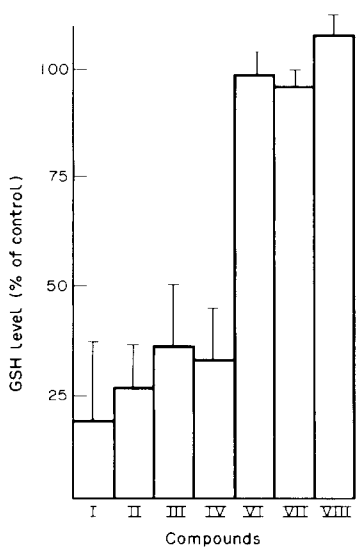

Fig. 3. Effect of PAR (I), the 3-monoalkyl-(II,III,IV) and the 3,5-dialkyl derivatives (VI,VII,VIII) on the GSH concentrations in hepatocytes at 1 hr incubation. Incubation of hepatocytes and determinations of GSH concentrations were performed as described in Materials and Methods. Results are expressed as means ± SD of 4 experiments.

Toxicity in hepatocytes

Hepatocytes from 3-MC pretreated rats were found to be more susceptible to toxicity induced by PAR and 3-methyl-PAR (II), when compared to hepatocytes from untreated animals (Table 3). Therefore, only freshly isolated hepatocytes from 3-MC pretreated rats were used in order to compare the toxicity of PAR and other alkyl-substituted derivatives. 3-t-Butyl-PAR (V) and 3,5-di-t-butyl-PAR (IX) were not studied because of their poor solubility.

Upon addition of PAR (0.5 mM) to hepatocytes from 3-MC pretreated rats, the cellular GSH level dropped to 20% of the control values (Fig. 3), whereas the LDH leakage increased more than five-fold (Fig. 4A). Equimolar concentrations of the 3-monoalkyl derivatives caused similar effects: GSH depletion (Fig. 3) and increased LDH leakage (Fig. 4B). In contrast, in the case of the 3,5-dialkyl derivatives neither GSH depletion nor an increase in LDH leakage was observed. Even after raising the concentrations of 3,5-dimethyl-PAR (VI) and 3,5-di-ethyl-PAR (VII) to 2.5 mM and 1.5 mM, respectively, GSH levels were not decreased and no increased LDH leakage was observed (Figs 3 and 4B).

DISCUSSION

The aim of the present study was to investigate the effects of 3-monoalkyl- and 3,5-dialkyl-sub-

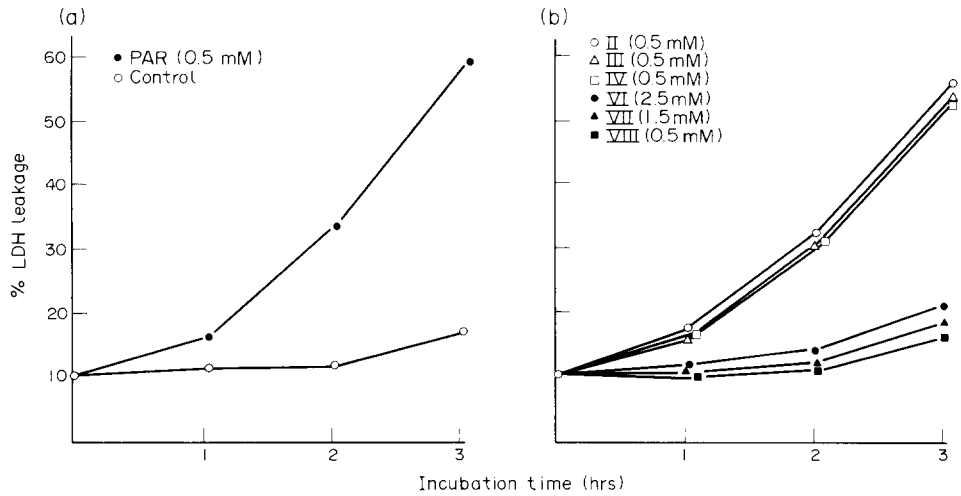

Fig. 4. The cytotoxicity of PAR (A), the 3-monoalkyl-(II,III,IV) and the 3,5-dialkyl-substituted derivatives (VI,VII,VIII) (B) in freshly isolated hepatocytes from 3-MC treated rats. Toxicity is expressed as per cent LDH leakage relative to total LDH activity present in cells. Incubations and LDH determination were performed as described in Materials and Methods. The results shown are means of five experiments (SD ≤ 10%).

stitution on the cytotoxicity of PAR. These types of substitution have been shown not to reduce the analgesic activity [15, 16]. The effects of alkyl-substitution on the cytotoxicity of PAR were studied at the level of interaction with, and oxidation by cytochrome P-450 to quinone imines, as well as the toxicity in hepatocytes.

As far as substrate binding to cytochrome P-450 is concerned, PAR and all derivatives interacted with cytochrome P-450 giving rise to reverse type I spectral changes. The manner of interaction of a substrate with cytochrome P-450 giving rise to a reverse type I spectral change is unknown [27, 30]. The finding of a strong correlation between the lipophilicity and

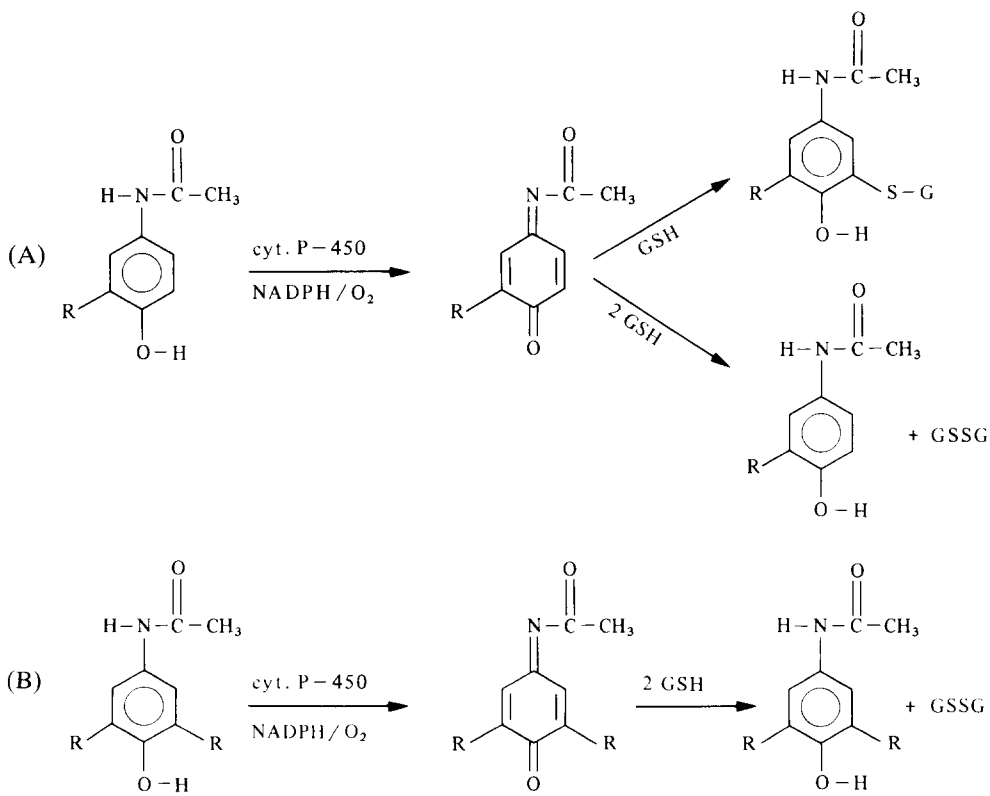

Fig. 5. Schematic representation of the proposed bioactivation of PAR and the 3-monoalkyl derivatives (A) and the 3,5-dialkyl derivatives (B) and the reactions of the formed *N*-acetyl-para-benzoquinone imines with GSH.

the logarithm of the K_s of PAR and its derivatives (Fig. 1A) suggests a hydrophobic nature for the interaction of these compounds with cytochrome P-450 and thereby supports the proposal that a reverse type I spectral change can be the result of substrate interaction with a binding site on the protein moiety, as in the case of a type I interaction [30].

PAR and all of the derivatives investigated appeared to be oxidized during microsomal incubation in the presence of NADPH as shown by reactions of the metabolites formed with GSH. In the case of PAR and the 3-monoalkyl derivatives, metabolites were formed whose reactions with GSH were identical to those of the corresponding synthetic NAPQI's with GSH, i.e. conjugation with GSH and oxidation of GSH to GSSG. The reactions of the metabolites from the 3,5-dialkyl derivatives resembled that of synthetically prepared 3,5-dimethyl-NAPQI, i.e. only oxidation of GSH to GSSG [28]. Although the reactive metabolites could not be detected directly, the secondary reactions with GSH indicate that PAR as well as the 3-monoalkyl- and the 3,5-dialkyl-substituted derivatives underwent biotransformation to their respective NAPQIs (Fig. 5).

The involvement of the cytochrome P-450 containing MFO system in the oxidation of PAR and its alkyl-substituted derivatives can be deduced from the requirement of NADPH and oxygen, the inhibition by SKF-525A, imidazole and ellipticine, well-known inhibitors of cytochrome P-450 mediated substrate oxygenation [29] (Table 2) and the differential effects of the cytochrome P-450 inducers 3-MC and PB. The actual effects of pretreatment of rats with 3-MC and PB on the cytochrome P-450 mediated oxidation of all derivatives of PAR investigated in the present study were similar to those earlier described for oxidation of PAR [13, 31]: enhanced product formation after induction by 3-MC and an unchanged or decreased product formation after induction by PB. This suggests that both PAR and its derivatives are oxidized by similar 3-MC-inducible cytochrome P-450 isoenzymes. In contrast to the strong correlation between log P and log K_s-values (Fig. 1A), the K_m-values of cytochrome P-450-mediated oxidation of PAR, its 3-monoalkyl- and 3,5-dialkyl derivatives did not correlate with their log P values (Fig. 1B). From this observation as well as from the absence of a relationship between K_m and the bulkiness of the substituents of the derivatives of PAR it can be concluded that the cytochrome P-450 mediated oxidation of PAR and its derivatives is primarily governed by other factors than lipophilicity and steric effects.

The toxicities of PAR and the 3-monoalkyl derivatives in the hepatocytes were found to be rather similar. These compounds were bioactivated to, most probably, NAPQIs which deplete cellular GSH by conjugation to GSH and possibly by GSH oxidation.

3,5-Dialkyl-substitution of PAR was found to prevent the depletion of GSH and the cytotoxic action of PAR. In principle, this effect might be ascribed to a difference in metabolism between these compounds and PAR. However, since alkyl-substitution at ortho-positions to the hydroxyl group of phenols has been shown to lead to a decrease in the rate of conjugative reactions [32, 33], one may assume that in the case of the 3,5-dialkyl derivatives of PAR saturation of conjugation would occur at the same or lower concentrations when compared to PAR. The involvement of liver cytosolic DT-diaphorase in the detoxification of the 3,5-dialkyl-NAPQIs, which could also account for the difference between the cytotoxicity of the 3,5-dialkyl derivatives and PAR is unlikely, since addition of the DT-diaphorase inhibitor dicumarol [34] did not lead to cytotoxicity of the 3,5-dialkyl-substituted derivatives. Moreover, synthetic 3,5-dimethyl-NAPQI and NAPQI were found not to be substrates for this cytosolic enzyme in a subcellular fraction of rat liver (data not shown). Therefore, the absence of toxicity of the 3,5-dialkyl derivatives of PAR can be directly related to apparently different toxic properties of the 3,5-dialkyl-NAPQIs when compared to NAPQI and 3-monoalkyl-NAPQIs.

As was shown in hepatic microsomal incubations, the reactive metabolites of the 3,5-dialkyl-derivatives of PAR only oxidized GSH to GSSG (Table 2, Fig. 5). However, no depletion of GSH was observed in hepatocytes. This may be explained by a rapid reduction of GSSG to GSH by cytosolic GSH-reductase. Recently, comparable behaviour of the hepatic GSH–GSSG redox-system has been observed in hepatocytes treated with other substituted quinones like adriamycin and menadione [35, 36]. In these cases, GSH depletion and cytotoxicity could only be observed after inhibition of GSH-reductase. One essential difference between PAR and the 3-monoalkyl derivatives, on the one hand, and the 3,5-dialkyl-substituted analogs, on the other hand, is therefore the irreversible depletion of cellular GSH by conjugation of GSH to the respective NAPQI metabolites of the former compounds and a reversible depletion of GSH (oxidation of GSH by the reactive metabolites, followed by reduction of GSSG by cellular GSH-reductase) in the case of the latter compounds. This difference is a result of the different chemical reactivity of the 3,5-dialkyl-NAPQIs and NAPQI towards thiols, which might also account for the observed difference between the cytotoxicity of 3,5-dialkyl-substituted derivatives of PAR and that of PAR in hepatocytes.

In the case of PAR, two processes, covalent binding of NAPQI to proteins [6, 7] and oxidative stress [8–10], have been proposed to be important steps in its hepatotoxic action. 3,5-Dialkyl-substitution of PAR may prevent the covalent binding to thiol groups of protein. This hypothesis is supported by the fact that irreversible conjugation of 3,5-dialkyl-NAPQIs to GSH was found to be absent. Furthermore, the reversible depletion of cellular GSH in the case of the 3,5-dialkyl-substituted derivatives leaves, in contrast to the action of PAR, the GSH–GSSG redox-system, a cellular defense system against oxidative stress [37, 38], relatively intact. Blocking of the reactive 3 and 5 positions of NAPQI appears to prevent its cytotoxicity. However, further studies are necessary in order to clarify fully the differences in mechanism of (toxic) actions of PAR and the 3,5-dialkyl derivatives of PAR.

As has been described previously, alkyl-substitution of PAR does not diminish the analgesic

activity [15, 16]. For that reason it is of importance to answer the question whether cytotoxicities of the derivatives of PAR determined in hepatocytes are comparable to hepatotoxicity *in vivo*. Preliminary *in vivo* data after oral dosage to mice indeed indicated that the 3-mono-alkyl derivatives were equally hepatotoxic as PAR and the 3,5-dialkyl-substituted derivatives were not hepatotoxic even at higher dosages. These findings, however, are in contrast to an equitoxicity of PAR and 3,5-dimethyl-PAR (VI), reported previously [39]. The cause of this discrepancy is as yet unclear.

In conclusion, it can be stated that alkyl-substitution of PAR does not dramatically affect its microsomal cytochrome P-450 mediated oxidation to reactive NAPQIs. When compared to PAR and 3-monoalkyl-substituted derivatives, 3,5-dialkyl-substitution changed the way of reacting of the formed NAPQIs with GSH. In these cases only reversible oxidation of GSH to GSSG was observed and no irreversible conjugation to GSH. In contrast to PAR and 3-monoalkyl-substituted derivatives, neither depletion of cellular GSH nor cytotoxicity in hepatocytes was observed for the 3,5-dialkyl derivatives. Based on the molecular mechanism of the toxic action of PAR, it is apparently possible to prevent the adverse effects of the drug by dialkyl-substitution. Interestingly, 3,5-dialkyl-substitution has been shown not to influence negatively the pharmacological activity of these compounds when compared to PAR [15, 16].

Acknowledgement—The authors would like to thank Dr J. C. Dearden for providing us with a number of paracetamol derivatives.

REFERENCES

1. E. M. Boyd and G. M. Bereczky, *Br. J. Pharmac.* **26**, 606 (1966).
2. M. Black, *Annu. Rev. Med.* **35**, 577 (1984).
3. J. A. Hinson, in *Reviews in Biochemical Toxicology*, Vol. 2 (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), p. 103. Elsevier, Amsterdam (1980).
4. J. A. Hinson, L. R. Pohl, T. J. Monks and J. R. Gillette, *Life Sci.* **29**, 107 (1981).
5. A. G. Streeter, D. C. Dahlin, S. D. Nelson and T. A. Baillie, *Chem.-Biol. Interact.* **48**, 349 (1984).
6. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
7. W. Z. Potter, S. S. Thorgeirsson, D. J. Jollow and J. R. Mitchell, *Pharmacology* **12**, 129 (1974).
8. R. L. Gerson, A. Casini, D. Gilfor, A. Serroni and J. L. Farber, *Biochem. biophys. Res. Commun.* **126**, 1129 (1985).
9. A. Wendel and S. Feuerstein, *Biochem. Pharmac.* **30**, 2513 (1981).
10. J. de Vries, *Biochem. Pharmac.* **30**, 399 (1981).
11. C. M. Steele, H. A. Masson, J. M. Battershill, G. G. Gibson and C. Ioannides, *Res. Commun. Chem. Path. Pharmac.* **40**, 109 (1983).
12. R. van de Straat, J. de Vries, H. J. R. de Boer, R. M. Vromans and N. P. E. Vermeulen, *Xenobiotica*, in press.
13. R. J. McMurtry, W. R. Snodgrass and J. R. Mitchell, *Toxic. appl. Pharmac.* **46**, 87 (1978).
14. D. Pessayre, J. C. Wandscheer, B. Cobert, R. Level, C. Degott, A. Batt, N. Martin and J. Benhamou, *Biochem. Pharmac.* **29**, 2219 (1980).
15. J. C. Dearden, J. H. O'Hara and M. S. Townsend, *J. Pharm. Pharmac.* **32**, 102p (1980).
16. J. C. Dearden, personal communication.
17. J. C. Dearden and J. H. O'Hara, *Eur. J. med. Chem.* **13**, 415 (1978).
18. H. W. Strobel and J. D. Dignam, in *Methods in Enzymology*, Vol. 52 (Eds. S. Fleischer and L. Packer), p. 89. Academic Press, New York (1978).
19. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
20. P. O. Seglen, *Exp. Cell. Res.* **82**, 391 (1973).
21. C. R. Jefcoate, in *Methods in Enzymology*, Vol. 52 (Eds. S. Fleischer and L. Packer), p. 258. Academic Press, New York (1978).
22. P. J. Hissin and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
23. P. Moldeus, *Biochem. Pharmac.* **27**, 2859 (1978).
24. L. J. Fischer, M. D. Green and A. W. Harman, *Drug Metab. Disp.* **13**, 121 (1985).
25. P. Moldeus, J. Högberg and S. Orrenius in *Methods in Enzymology*, Vol. 52 (Eds. S. Fleischer and L. Packer), p. 60. Academic Press, New York (1978).
26. G. L. Ellman, *Archs Biochem. Biophys.* **82**, 70 (1959).
27. J. B. Schenkman, S. G. Sligar and D. L. Cinti, in *Hepatic Cytochrome P-450 Monooxygenase System* (Eds. J. B. Schenkman and D. Kupfer), p. 587. Pergamon Press, Oxford (1982).
28. G. M. Rosen, E. J. Rauckman, S. P. Ellington, D. C. Dahlin, J. L. Christie and S. D. Nelson, *Molec. Pharmac.* **25**, 151 (1984).
29. B. Testa and P. Jenner, *Drug Metab. Rev.* **12**, 1 (1981).
30. G. G. Gibson and P. P. Tamburine, *Xenobiotica* **14**, 27 (1984).
31. C. Ioannides, C. M. Steele and D. V. Parke, *Toxic. Lett.* **16**, 55 (1983).
32. H. G. Bray, B. G. Humphris, W. V. Thorpe, K. White and P. B. Wood, *Biochem. J.* **52**, 419 (1952).
33. H. G. Bray, B. G. Humphris, W. V. Thorpe, K. White and P. B. Wood, *Biochem. J.* **52**, 416 (1952).
34. C. Lind, P. Hochstein and L. Ernster, *Archs Biochem. Biophys.* **216**, 178 (1982).
35. J. R. Babson, N. S. Abell and D. J. Reed, *Biochem. Pharmac.* **30**, 2299 (1981).
36. J. L. Farber and P. E. Starke, *J. biol. Chem.* **260**, 86 (1985).
37. P. Moldeus and B. Jerström, in *Functions of Glutathione, Biochemical, Physiological, Toxicological and Clinical Aspects* (Eds. A. Larsson, S. Orrenius, A. Holmgren and B. Mannervik), p. 99. Raven Press, New York (1983).
38. D. Dimonte, G. Bellamo, H. Thor, P. Nicotera and S. Orrenius, *Archs Biochem. Biophys.* **235**, 343 (1984).
39. C. R. Fernando, I. C. Calder and K. N. Ham, *J. med. Chem.* **23**, 1153 (1980).