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EFFECT OF SPIN TRAPS IN ISOLATED RAT HEPATOCYTES AND LIVER MICROSOMES

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Abstract—Spin traps are increasingly employed in the detection of free radicals in biological systems, including liver microsomes and isolated hepatocytes.

Two spin traps phenyl-*t*-butyl nitrone (PBN) and 4-pyridyl-l-oxide-*t*-butyl nitrone (4-POBN) have been tested for their effects on hepatocyte viability and mixed-function oxidase activity.

High concentration of PBN but not of 4-POBN proved to moderately affect liver cell integrity, without interfering with intracellular ATP or cytochrome P-450 content.

PBN also decreased hepatocyte GSH content, probably as the result of its metabolism to benzaldehyde. The two spin traps were found to inhibit aminopyrine demethylase and ethoxycoumarin deethylase activity in hepatocytes and microsomes. At low concentrations (1–5 mM) PBN enhanced aniline hydroxylase while high concentrations of the spin trap inhibited this activity.

The inhibition of the monooxygenase system was not caused by damage of microsomal enzymes, but rather by competition with other substrates for the binding to the haemoprotein.

The effects of spin traps on mixed function oxidase systems should be taken into account when evaluating the results of spin trapping experiments.

Spin traps were first used in biological systems by Lai and Piette [1] and Ingal and coworkers [2] in attempts to detect, respectively, hydroxyl and trichloromethyl radicals. Since then, the technique of spin trapping has been successfully employed in the detection of highly unstable free radicals produced by liver microsomes and isolated hepatocyte suspensions and even in animal liver *in vivo* [3–5].

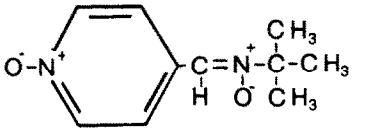
The possibility of trapping free radical intermediates largely depends upon the competition between the rates of spin adduct formation and those of other reactions of the radical under study [6]. This limitation may become especially significant when the spin trapping technique is employed in biological systems because of the heterogeneous composition of the incubation mixture and the often low rate of radical production by enzymatic systems [4]. To increase the yield of the free radical adduct, spin traps are used in the highest possible concentration, often in excess of any other constituent of the incubation mixture.

Reports concerning the adverse effects of spin traps have been very scattered [7–12] and only recently has the problem been brought into focus [4, 12]. The data available indicate that the nitrone spin trap 5,5 dimethyl-pyrroline-*N*-oxide (DMPO) at concentrations up to 80 mM does not affect NADPH : cytochrome *c* reductase activity in rat liver microsomes [7]. At lower concentrations (e.g. 30 mM), DMPO is without effects on aminopyrine demethylation and aniline hydroxylation [8], but slightly reduces benzphetamine-*N*-demethylation [9].

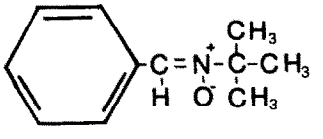
Under similar conditions, PBN and alpha(4-pyridyl-1-oxide)-*N*-*t*-butyl nitrone (4-POBN) are, however, effective inhibitors of benzphetamine demethylation, reducing it by approximately 75% and 35%, respectively [9].

Methylnitroso propane (MNP) and PBN at 5 mM concentration also inhibit aminopyrine demethylation in liver microsomes [10], while PBN has been found to reduce the metabolism of alkyl-hydrazines [11].

The possible mutagenic activity of PBN, 4-POBN and DMPO has also been investigated and all the compounds showed no effect [13].



4-POBN



PBN

Fig. 1. Chemical structures of the spin traps phenyl-*t*-butyl nitrone (PBN) and alfa(4-pyridyl-1-oxide)-*t*-butyl nitrone (4-POBN).

In the present study we have investigated the effects produced by PBN and 4-POBN (Fig. 1) on isolated hepatocyte integrity and mixed-function oxidase activity.

Attention has been focused on these two trapping agents because they are particularly suitable for the detection of radical intermediates produced during drug metabolism and because they are frequently employed in experiments with liver microsomes, isolated hepatocytes and in the whole animal [3–5, 10].

MATERIALS AND METHODS

Phenyl-*t*-butyl nitrone (PBN), alpha(4-pyridyl-1-oxide-*N*-*t*-butyl nitrone (4-POBN) and ethoxycoumarin were obtained form Aldrich-Europe (Beerse, Belgium). Collagenase, glucuronydase: arylsulphatase, glucose-6-phosphate dehydrogenase, NADP and glucose-6-phosphate were supplied by Boehringer-Biochemia (Mannheim, F.R.G.). Other reagents were of analytical grade and were obtained from Merck (Darmstadt, F.R.G.).

Male Wistar rats (200–300 g b.wt) were pretreated with phenobarbital 0.1% in the drinking water for at least one week before use.

Isolated rat hepatocytes were prepared by collagenase perfusion of the liver as previously described [14] and the incubation conditions were analogous to those employed for spin trapping experiments [10].

Liver cell integrity was estimated by monitoring the release in the cell-free supernatant of lactate dehydrogenase (LDH) and aspartate aminotransferase (ASAT) according to [14], using a Boehringer-Biochemia Kit (Boehringer, Mannheim, F.R.G.). Hepatocyte ATP content was measured enzymatically using Boehringer Test-Combination kit. Reduced glutathione (GSH) was measured with the Ellman's reagent as previously described [15].

Aminopyrine demethylase activity in the hepatocytes was determined through the release of $^{14}[C]$-CO_2 from (dimethylamino ^{14}C)-aminopyrine. For these experiments radioactive aminopyrine (1 mM; specific activity 0.1 mCi/mmol) (Radiochemical Centre, Amersham, U.K.) was added to 1 ml hepatocyte suspension and incubated with or without spin traps in flasks sealed with rubber stoppers. The reaction was terminated by adding 0.5 ml 5 N H_2SO_4 through the stopper and CO_2 was collected on filter paper soaked with 1 N KOH, placed in plastic vials inside the flasks. The radioactivity trapped was then measured by adding the filters to 5 ml Beckman Ready-Solve NA scintillation fluid and counting them with an LKB Rank-Beta counter.

The procedure described by Orton *et al.* [16] was used, with some modifications, for monitoring ethoxycoumarin deethylase activity in hepatocytes. Isolated liver cells were incubated for 15 min with 0.08 mM ethoxycoumarin and in the presence of either PBN or 4-POBN. At the end of the incubation, 0.5 ml of cell-free supernatant was added to the same volume of 0.5 M acetate buffer pH 5 containing 50 μl glucuronidase: arylsulphatase solution and incubated for 4 hr at 37°. The product, 7-hydroxycoumarin was then extracted with 10 ml ethyl-ether containing 1.5% amyl alcohol and 5 ml of the extract were dried under a stream of nitrogen and resuspended with 3 ml NaOH–glycine buffer pH 10.4 immediately before fluorimetric analysis. The instrument was calibrated with 7-hydroxycoumarin standard in the same buffer.

Liver microsomes were prepared as previously described [17] and washed once by resuspension and recentrifugation in 0.1 mM Tris–HCl buffer pH 7.4.

Cytochrome P-450 and NADPH: cytochrome *c* reductase were determined as described in [17]. Binding spectra of PBN and 4-POBN to microsomal cytochrome P-450 were determined according to Jefcoate [18].

Aminopyrine demethylase was measured by incubating microsomes (approx. 2 mg protein), Tris–HCl buffer (pH 7.4) (0.1 mmol), $MgCl_2$ (12.8 μmol) glucose-6-phosphate (12.8 μmol), NADP (0.8 μmol), glucose-6-phosphate dehydrogenase (0.7 units) and aminopyrine (10 μmol) in a final volume of 2 ml, at 37° in the dark for 10 min. At the end of the incubation, 0.25 ml $ZnSO_4$ (25%) and 0.25 ml $Ba(OH)_2$ (saturated) were added and formaldehyde was estimated in the protein-free supernatant by the method of Nash [19].

Aniline hydroxylase activity was measured using the same incubation medium, replacing aminopyrine

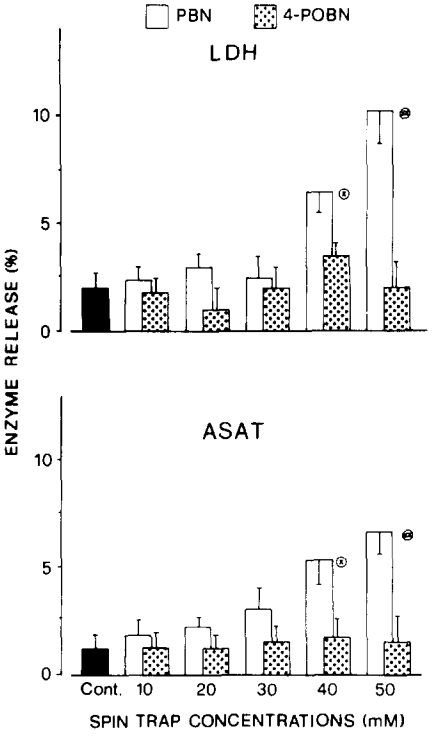


Fig. 2. LDH and ASAT leakage from isolated hepatocytes exposed to increasing concentrations of PBN and 4-POBN. Liver cell suspensions (7.5×10^6 cells/ml) were incubated for 60 min at 37° in the presence of various spin trap concentrations and the enzyme activity was estimated in the cell-free supernatant. The values are expressed as percent of the total enzyme content measured after cell lysis with 0.5% Triton X100. The results are means of three different experiments ±S.D. Statistical significance was assessed by Student's *t*-test: × $P < 0.05$; ×× $P < 0.01$ respect to controls.

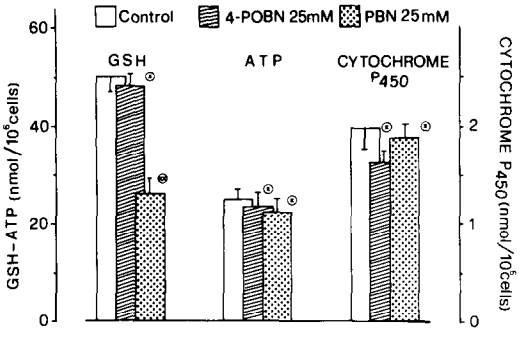


Fig. 3. Effects of spin traps on cellular content of GSH, ATP, and cytochrome P-450 of isolated hepatocytes. Isolated liver cells (7.5×10^6 cells/ml) were incubated for 30 min at 37° in the presence of 25 mM of either PBN and 4-POBN. The values are means of three separate experiments ±S.D. Statistical significance was assessed by Student's *t*-test: × not significant; ×× $P < 0.01$.

with aniline sulphate (1 mM final concentration) and stopping the incubation (15 min) with 1 ml 20% (w/v) trichloroacetic acid. To 1 ml of protein-free supernatant was added 1 ml of alkaline phenol solution (1% phenol w/v in 0.1 N NaOH) and 1 ml Na_2CO_3 (2 M). The resulting blue colour was measured at 635 nm after 30 min at room temperature and compared to a calibration curve obtained with standard *p*-aminophenol.

Ethoxycoumarin deethylase activity was determined using the same microsomal incubation as above mentioned containing instead 0.1 mM ethoxycoumarin. The incubation (15 min) was stoped with 10 ml ethyl-ether containing 1.5% amyl alcohol and the ether extract was processed as reported for hepatocyte experiments.

Proteins were determined by the method of Lowry *et al.* [20].

RESULTS

The effects exerted by increasing concentrations of spin traps on cellular integrity of isolated hepatocytes from phenobarbital-treated rats have been monitored by the leakage of lactate dehydrogenase (LDH) and aspartate aminotransferase (ASAT).

The incubation of isolated hepatocytes with PBN, at concentrations up to 50 mM, moderately affects cellular integrity only at the highest concentrations, while 4-POBN is without effect over the same concentration range (Fig. 2).

Since most of the spin trapping studies in isolated liver cells were performed using a concentration of 25 mM [10, 21, 22], further experiments have been carried out to evaluate the effects of this dose of spin trap.

When incubated with isolated hepatocytes both PBN and 4-POBN at 25 mM cause a not significant reduction ($P > 0.5$) in cellular ATP content, while a 50% loss in intracellular glutathione (GSH) content is evident only in hepatocytes receiving PBN (Fig. 3).

Time-course experiments on this latter aspect show that GSH decreases steadily with time, but without significant differences between the various (5, 10, 25 mM) concentrations of PBN tested (not shown). Since PBN does not spontaneously react with GSH, it is likely that depletion of the tripeptide is mediated by some metabolite produced during the biotransformation processes. Table 1 shows that cytochrome P-450 inhibition by 0.5 mM SKF 525A prevents by 67% and 58% the GSH loss induced by, respectively, 5 and 25 mM PBN.

Thus it is possible that cytochrome P-450-catalyzed oxidation of the C=N double bond in the PBN molecule produces benzaldehyde, which then reacts with GSH. In accordance with this view 4-POBN, which differs from PBN in having a pyridyl-*N*-oxide group in the place of the phenyl group does not cause GSH depletion.

The effects of PBN and 4-POBN on the mixed-function oxidase activity of isolated hepatocytes have been investigated by monitoring aminopyrine demethylation and ethoxycoumarin-*O*-deethylation. Both monooxygenase activities are strongly reduced by PBN in a concentration-dependent fashion, while 4-POBN has only modest effects (Fig. 4). At 25 mM, PBN and 4-POBN decrease aminopyrine demethylation by 57% and 19%, respectively, and ethoxycoumarin deethylation by 80% and 28%, respectively.

These inhibitions cannot be ascribed to damage of cytochrome P-450, since neither spin trap significantly modifies the haemoprotein content of hepatocytes (Fig. 3).

In order to investigate in detail the mechanisms responsible for inhibition of drug metabolism, further experiments have been performed with phenobarbital-induced rat liver microsomes.

Table 1. Effect of the cytochrome P-450 inhibitor SKF 525A on the depletion of glutathione induced by phenyl-butyl nitrone (PBN) in isolated rat hepatocytes

Treatment	GSH (nmol/10^6 cells)	Decrease (%)
None	52.2 ± 4.6	—
PBN 5 mM	23.1 ± 5.1	54%
+ SKF 525A 0.5 mM	41.3 ± 3.7	18%
PBN 25 mM	27.8 ± 4.2	47%
+ SKF 525A 0.5 mM	43.4 ± 2.8	20%

The values refer to hepatocyte suspensions (7.5×10^6 cells/ml) incubated for 15 min at 37°, and are means of three separate experiments ±S.D.

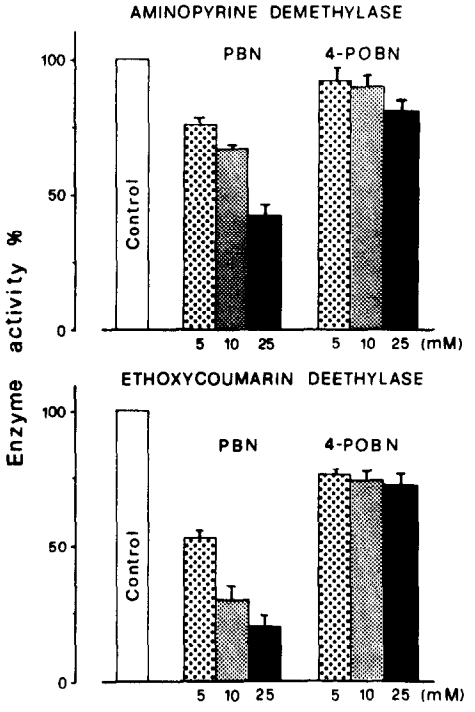
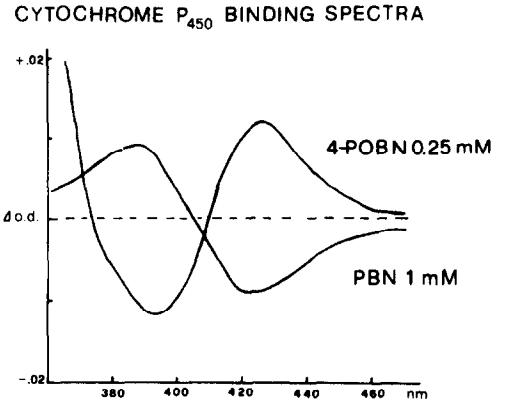


Fig. 4. Inhibition of mixed function oxidase activity in isolated hepatocytes exposed to PBN and 4-POBN. The results are expressed as percent of the enzymatic activities in hepatocyte suspensions (7.5×10^6 cells/ml) incubated 15 min at 37° without spin traps. In these conditions aminopyrine demethylase activity was 48.3 ± 3.5 nmol/10^7 cells/15 min, while ethoxycoumarin deethylase activity was 66.9 ± 7.2 nmol/10^7 cells/15 min. The values represent the means of four separate experiments ±S.D.



	λ_{max}	λ_{min}
4-POBN	426	394
PBN	388	423

Fig. 5. Cytochrome P-450 binding spectra of PBN and 4-POBN in phenobarbital-induced rat liver microsomes.

When added to microsomal suspension, both PBN and 4-POBN interact with cytochrome P-450 giving substrate binding spectra, evidenced by difference spectroscopy [18]. The spectrum observed in the presence of PBN has a peak at 388 nm and a trough at 423 nm, typical of the so-called Type I substrates (Fig. 5). On the other hand, 4-POBN gives spectral changes characterized by a peak at 426 nm and a trough at 394 nm, indicative of a Type II substrate (Fig. 5). The apparent dissociation constants are 0.34 mM for PBN and 0.64 mM for 4-POBN.

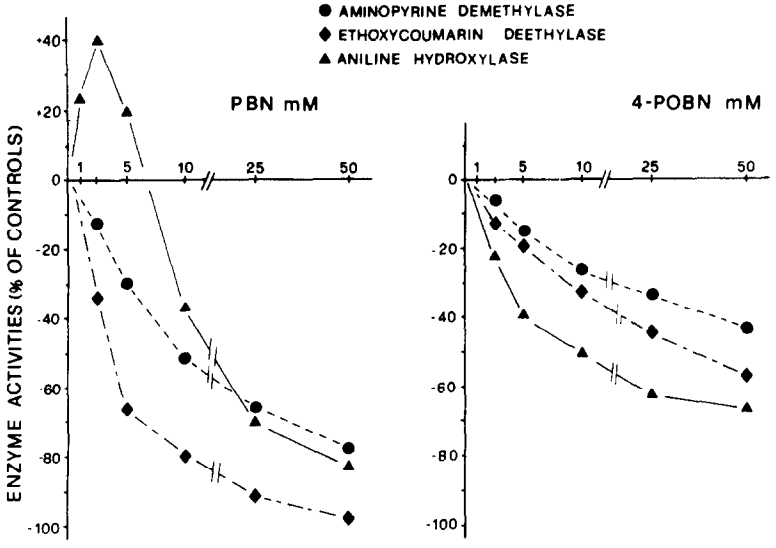


Fig. 6. Effects of PBN and 4-POBN on mixed function oxidase activity of microsomes. The results are expressed as percent of the enzymatic activities of microsomal preparations incubated without spin traps and corresponding, respectively, to 15.7 ± 4.1 nmol/mg protein/min for aminopyrine demethylation; 1.19 ± 0.31 nmol/mg protein/min for ethoxycoumarin deethylation; 1.26 ± 0.41 nmol/mg/min for aniline hydroxylation. The values represent the means of 4–6 separate determinations. For each experimental point standard deviations were ranging from ±3% to ±7%.

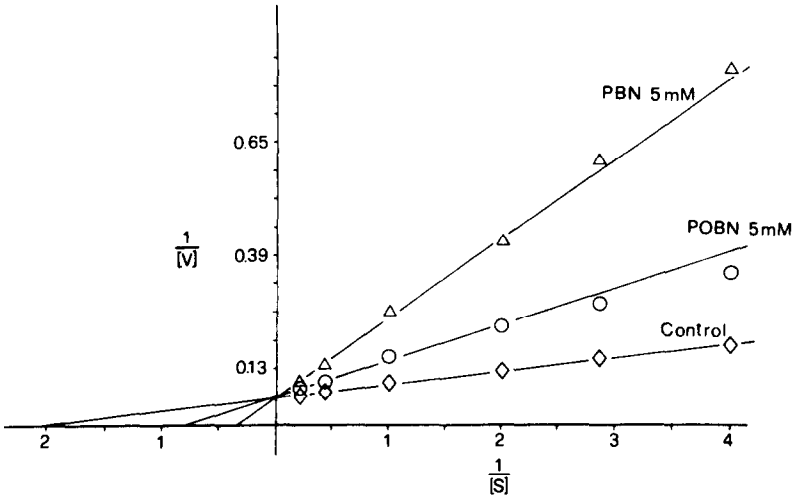


Fig. 7. Lineweaver–Burk plot of PBN and 4-POBN induced inhibition of aminopyrine demethylase activity in liver microsomes. The values for V_{max} in controls and in the presence of 5 mM PBN or 5 mM 4-POBN were 15.7, 17.0 and 16.3 nmol/mg/min respectively; the values for K_m were 0.48, 2.38 and 0.99 mM respectively (means of two experiments).

Microsomal mixed-function oxidase activity, exemplified by aminopyrine demethylation, aniline-*p*-hydroxylation and ethoxycoumarin-*O*-deethylation are all affected by the two spin traps (Fig. 6). A notable exception is the enhancement of aniline hydroxylase activity found at low concentrations (5 mM and below) of PBN; higher concentrations, instead, inhibited aniline hydroxylation. In microsomal suspensions 4-POBN is approximately twice as active as an inhibitor compared to cell suspensions and in both systems PBN is more effective than 4-POBN.

In liver microsomes, as in the isolated hepatocytes, PBN and 4-POBN at concentrations up to 50 mM do not significantly influence the cytochrome P-450 content, nor do they affect the NADPH: cytochrome c reductase activity (not shown). Thus, the observed inhibition of mixed-function oxidase activity is not likely to be caused by enzymatic damage.

Lineweaver–Burk analysis of the spin trap effect on aminopyrine demethylation show that PBN and 4-POBN act by a competitive mechanism (Fig. 7). The same figure reports the changes in K_m and V_{max} of the above enzymatic activity in the presence of 5 mM spin trap concentration and show a two- and five-fold increase of K_m values in the presence of, respectively, 4-POBN and PBN.

These variations correlate well with the apparent affinities (K_s) of the two spin traps with cytochrome P-450; in fact, PBN which has a K_s approximately double that of 4-POBN, is also twice as effective as an inhibitor.

DISCUSSION

The cellular integrity of isolated hepatocyte suspensions exposed to spin trapping agents has been evaluated by exploring both plasma membrane integrity and metabolic competence of hepatocytes [23]. On this basis, spin trapping agents appear to be well tolerated by isolated rat hepatocytes and only PBN slightly reduces cell viability, when used at relatively high concentrations.

However, the exposure of hepatocytes to spin trap concentrations suitable for the detection of free radicals causes some undesirable effects. PBN rapidly depletes intracellular GSH content, probably as a result of its metabolism by the mixed function oxidase system. Increasing the concentration of PBN from 5 to 25 mM does not influence the decrease of hepatocyte GSH content probably because cytochrome P-450 is already saturated at the lowest concentration used. According to this interpretation PBN shows a K_s value for the haemoprotein of 0.34 mM.

The GSH depletion may potentially interfere with spin trapping of free radical metabolites, because it could affect hepatocyte viability [24].

Furthermore, depletion of GSH stimulates lipid peroxidation by reducing a defence mechanism against oxidizing agents [24], thus generating radical species that may complicate the interpretation of ESR spectra. However, the PBN-induced decrease in GSH reported here might not be sufficient to generate such artefacts.

PBN and 4-POBN also act as substrates for cytochrome P-450, thereby inhibiting monooxygenase activities through a competitive mechanism. Their effects correlate well with the relative dissociation constants for cytochrome P-450; the highly lipophilic PBN binds strongly to the haemoprotein (K_s 0.34 mM) and effectively inhibits both aminopyrine and ethoxycoumarin metabolism. 4-POBN, on the other hand, is less tightly bound (K_s 0.64 mM) and is much less active as an inhibitor.

Similar findings have been recently reported by Augusto *et al.* [9], although the K_s for PBN they find (0.15 mM) is lower than that reported here.

When analysed by difference spectroscopy, PBN and 4-POBN give qualitatively different binding spectra with cytochrome P-450. PBN elicits a Type I spectrum, representative of an interaction with the

substrate-binding site of the cytochrome, while 4-POBN gives a Type II spectral change, probably resulting from coordination of the *N*-oxide group to the haem-iron [18].

Probably related to the differences in the chemical structure of the two spin traps is the stimulation given by PBN, but not by 4-POBN, on the aniline hydroxylase activity of microsomes. It has been postulated that interaction with the lipophilic environment of cytochrome P-450 could be the reason for the stimulatory effect, that is common to several other unrelated chemicals such as acetone and various nitrogen heterocycles [25, 26].

The effects exerted by spin traps on the drug metabolizing system may, of course, greatly influence free radical detection by reducing the rate of xenobiotic activation. In this respect, the spin traps with lower binding affinity for cytochrome P-450 may be expected to give less inhibition and to be more efficient traps than those with high binding capacity. This, however, is not always true, since Augusto and coworkers [9] have noticed that DMPO, which binds weakly to cytochrome P-450 and does not significantly affect monooxygenase activity [9], is quite ineffective as a spin trap for radicals produced by the mixed function oxidase system [9, 11]. A possible explanation for these findings could be that, being a hydrophilic compound, DMPO is present in low amounts in the lipophilic environment of cytochrome P-450, so its chances to trap radicals are minimal.

On the other hand, PBN has been found to be a good spin trap for haloalkane-derived radicals [5, 10, 21, 22], even though it is a strong inhibitor of the drug metabolizing enzymes. Nevertheless, it is difficult to establish if the decrease in covalent binding of CCl_4 to proteins observed in the presence of PBN [10] is due to the trapping of the radical metabolites or to an inhibition of haloalkane metabolism. PBN has been reported to interfere with the free radical activation of some alkyl-hydrazines [9], but it is possible that different cytochrome P-450 isoenzymes may be involved in halogenated hydrocarbon metabolism.

In conclusion, the interaction of spin traps with the monooxygenase system at least indicates that they are able to reach the site of free radical formation and may thus be effective trapping agents for short-lived radicals. None the less, in the case of negative spin trapping results, potential inhibition of the activating enzymes by spin traps should be taken into account before ruling out the presence of free radicals.

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