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CYTOCHROME P450 PRODUCT COMPLEXES AND GLUTATHIONE CONSUMPTION PRODUCED IN ISOLATED HEPATOCYTES BY NORBENZPHETAMINE AND ITS *N*-OXIDIZED CONGENERS

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Abstract—Incubation of liver microsomes from phenobarbital-treated rats with norbenzphetamine (I) and its two *N*-oxidized metabolites, *N*-hydroxynorbenzphetamine (II) and the corresponding nitrone (III), in the presence of NADPH and molecular oxygen, gave rise to the formation of cytochrome P450 product complexes characterized by maximal absorbance at 455 nm. The complex forming activity increased in the order I, II and III, with the nitrome (III) exhibiting an activity of about 60 per cent of that of *N*-hydroxyamphetamine (IV). The same relative order of complex forming activity was seen also when incubations were performed with hepatocytes isolated from phenobarbital-treated rats, but the complex formation was less rapid as well as less extensive. Liver tissues from untreated rats exhibited the same complex forming pattern, but with considerably lower activity. Incubations of hepatocytes with I, II and III caused a decrease in the cellular level of reduced glutathion (GSH) and II and III caused the most significant drops in the GSH level. The decrease in GSH was enhanced in hepatocytes from phenobarbital-treated rats. *N*-hydroxyamphetamine had no effect on the cellular GSH level and the amount of oxidized glutathione (GSSG) was unaffected by addition of I–IV. It is suggested that *N*-hydroxyamphetamine (IV), formed by hydrolysis of the nitrone (III), is the ultimate substrate for the reaction leading to complex formation. The results indicate that the nitrone (III) is a common intermediate in the reactions leading to the interaction with GSH and cytochrome P450 complex formation and by reacting with III, GSH decreases the concentration of cytochrome P450-binding norbenzphetamine metabolites in the hepatocyte.

In 1973 Werringloer and Estabrook [1] reported on the formation of a cytochrome P450 product complex during NADPH-dependent metabolism of benzphetamine by liver microsomes. They concluded that further metabolic conversion of a metabolite of benzphetamine was most likely the cause for the generation of the cytochrome P450 adduct. Since this report, various other phenylalkylamines [2] have been shown to form similar complexes, characterized by a light absorption maximum at about 455 nm, and Franklin [3] subsequently found *N*-hydroxyamphetamine (IV) to be the most potent substrate in this respect. In two recent publications [4,5] we presented evidence suggesting *N*-oxidation to be a prerequisite for cytochrome P450 complex formation with phenethylamines. Although the oxidation state of the nitrogen in these complexes has not been unequivocally established, either the nitroso compound [4–6] or the nitroxide radical [4,5] can be suggested as distinct possibilities for the reaction.

We recently also reported [7] that norbenzphetamine (I) produces a cytochrome P450 product complex when metabolized by hepatocytes obtained from phenobarbital-treated rats. In these studies, when utilizing isolated liver cells, a decrease in the cellular level of glutathione (GSH) could be observed, suggesting consumption of GSH during the metabolism of the drug. The results indicated an interaction between complex formation and GSH consumption and suggested that further metabolism by a GSH-linked pathway may act for decrease in the concentration of cytochrome P450 binding norbenzphetamine metabolites in the hepatocytes. Consequently, since *N*-oxidation seems to be a necessity for complex formation, *N*-oxidized derivatives of norbenzphetamine (I) can also be expected to cause GSH consumption.

Evidence has been presented [8] for the enzymatic formation of *N*-benzyl-*N*-hydroxyamphetamine (II) and the nitrone (*N*-benzylidene-1-phenyl-2-propylamine-*N*-oxide, III) during the *in vitro* metabolism of norbenzphetamine (I) (Fig. 1) and this communication reports on cytochrome P450 product complexes and GSH consumption produced in isolated hepatocytes by I, II and III. The complexes obtained with the hepatocytes are also compared with those produced by liver microsomes.

MATERIALS AND METHODS

(±)-*N*-benzyl-1-phenyl-2-propylamine (I) was prepared from amphetamine and benzaldehyde as described by Schaeffer *et al.* [9]. *N*-benzyl-*N*-hydroxyl-1-phenyl-2-propylamine (II) and *N*-benzylidene-1-phenyl-2-propylamine-*N*-oxide (III) were synthesized

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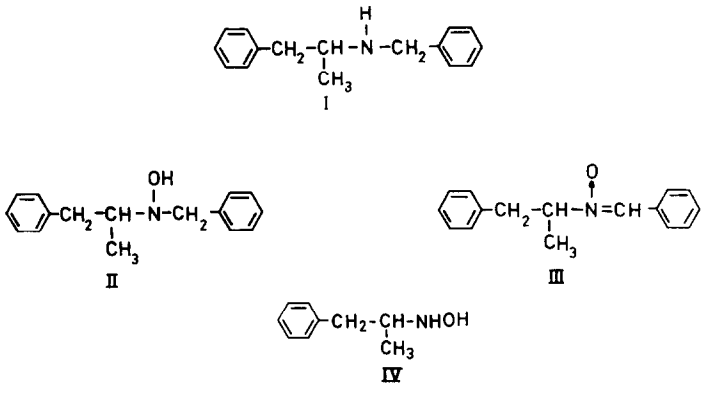


Fig. 1. Chemical structure of norbenzphetamine (*N*-benzyl-1-phenyl-2-propylamine, I), *N*-hydroxynorbenzphetamine (*N*-benzyl-*N*-hydroxy-1-phenyl-2-propylamine, II), the nitrone (*N*-benzylidene-1-phenyl-2-propyl-amine *N*-oxide, III, and *N*-hydroxyamphetamine (*N*-hydoxy-1-phenyl-2-propylamine, IV).

according to Beckett *et al.* [10]. Compound II was isolated as its acid oxalate and the correct structures of II and III were established by their melting points and i.r. and NMR spectra, which were all in agreement with those earlier reported [10]. The synthesis of *N*-hydroxyamphetamine (IV) is described in the cited previous publication [11].

Microsomes and hepatocytes were isolated from livers of adult Sprague–Dawley rats (200–250 g). The rats were either untreated or received phenobarbital (four daily i.p. injections of 80 mg/kg) prior to sacrifice. Microsomes were isolated according to Ernster *et al.* [12] and hepatocytes as described by Högberg and Kristofferson [13].

Unless otherwise stated, the hepatocytes (10^6 cells/ml) were incubated in a Waymouth medium (MB 752/1, Gibco-Biocult) supplemented with 17.5% horse serum, 25 mM HEPES, 500 IU/ml penicillin and 10 IU/ml heparin (referred to as a Waymouth complete medium), under a stream of 95% O_2–5% CO_2 at 37° in rotating flasks. The number of viable cells was estimated periodically during the incubation by a latency test for lactic dehydrogenase [13].

For determination of GSH in the cells, a 1 ml aliquote of the incubation mixture was centrifuged at 300 rpm for 2 min and the pellet and supernatant were separated. After addition of 1 ml 6.5% trichloroacetic acid to the pellet and subsequent centrifugation at 2,000 rpm for 15 min, a 0.5 ml aliquot of the supernatant was subjected to analysis according to Saville [14]. Oxidized glutathione (GSSG) was determined fluorophotometrically [15] as follows: isolated hepatocytes, suspended in Krebs–Henselite buffer containing 2% bovine serum albumin (Krebs–albumin buffer), were centrifuged at 300 rpm for 2 min and the supernatant and pelleted cells were separated. After addition of trichloroacetic acid and subsequent centrifugation, an extract (0.5 ml) from both supernatant and cell pellet was recovered. To 0.5 ml extract was added 0.2 ml *N*-ethylmaleimide and the mixture was allowed to stand at 25° for 30 min, whereupon 4.1 ml 0.1 N NaOH and 0.25 ml 0.1% *o*-phtalaldehyde were added. After 15 min the fluorescence at 420 nm was determined (activation at 350 nm). In one set of experiments, simultaneous determination of GSH and GSSG was performed using Krebs–albumin buffer as the cell suspension medium, since the Waymouth medium interfered with the fluorometric determination of GSSG.

The formation of cytochrome P450 product complexes was determined by difference spectroscopy using an Aminco DW-2 spectrophotometer. For the spectral studies, isolated hepatocytes (10^6 cells), suspended in Krebs–albumin buffer, were incubated at 37° using a mixture of 95% O_2 –5% CO_2 as the gas phase. Similarly, microsomes (0.4 mg protein/ml) were incubated at 37° in 50 mM Tris–HCl buffer, pH 7.4, containing 14 mM KCl, 10 mM $MgCl_2$ and NADPH generating system. The substrates were dissolved in H_2O or EtOH and 100 μl of the solution was added to the sample cuvette, while the same amount of pure solvent was added to the reference cuvette. Semi-anaerobic experiments were conducted at37° in the presence of glucose and glucose oxidase. Microsomes from phenobarbital-treated rats and the glucose oxidase system were incubated in Thunberg cuvettes for 10 min, the air was evacuated and complex formation was initiated by addition of NADPH. Protein concentration [16] and cytochrome P450 [17] were determined according to standard procedures. The cell experimental data are taken from a series of experiments, performed with different batches of isolated hepatocytes.

RESULTS

As reported earlier [7] norbenzphetamine (I) is a relatively poor substrate for cytochrome P455 complex formation in hepatocytes. Of the various benzphetamine derivatives investigated in the present study, the nitrone (III) was the best substrate for the reaction leading to complex formation in tissues obtained from both phenobarbital-treated and control rats (Figs. 2 and 3). In hepatocytes isolated from phenobarbital-treated rats (Fig. 2B), III exhibited an activity of about 60 per cent of that of *N*-hydroxyamphetamine (IV), the latter being used as a standard. The hydroxylamine (II) was less active than the nitrone (III) but considerably more active than norbenzphetamine (I). In relation to *N*-hydroxyamphetamine (IV), compounds I, II and III all showed a higher complex forming activity in microsomal preparations from phenobarbital-treated rats (Fig.

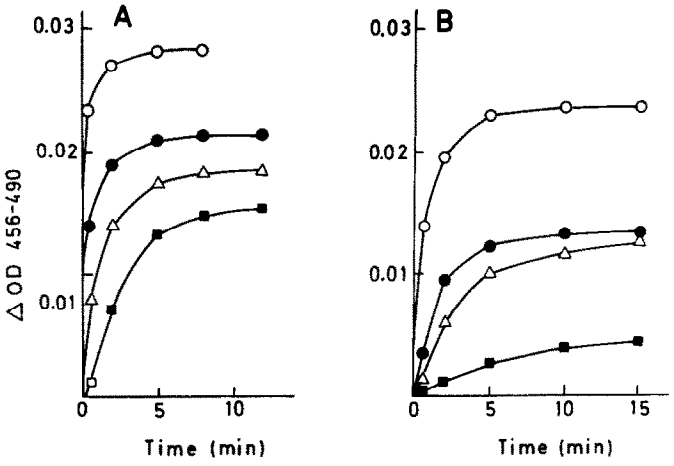


Fig. 2. Cytochrome P450 product complex formation by substrates I–IV in microsomes (A) and hepatocytes (B) isolated from phenobarbital-treated rats. Microsomes (0.8 μM of cytochrome P450) or hepatocytes (10^6 cells) were incubated in the presence of *N*-hydroxyamphetamine (IV, ○——○), the nitrone (III, ●——●), *N*-hydroxynorbenzphetamine (II, △——△) and norbenzphetamine (I, ■——■). The substrate concentrations were 100 μM. One representative experiment out of six. The standard deviation was less than 16 per cent [5].

2A) than in hepatocytes from similarly treated animals (Fig. 2B). The difference was most pronounced for the amine (I, ratio 3.3:1), while the hydroxylamine (II) and the nitrone (III) exhibited smaller and similar differences (ratios 1.2:1 and 1.3:1, respectively). The latter differences cannot, however, be regarded as significant, as with the hepatocytes, complex formation varied ± 10%, depending on the cell preparation.

Figure 3 shows the time course of P450 product complex formation during metabolism of I, II, III and IV in liver microsomes isolated from control rats. As compared with microsomes from phenobarbital-treated rats, a remarkably slower rate of complex formation was observed and the most striking difference

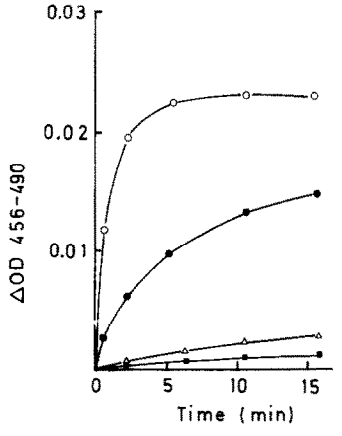


Fig. 3. Cytochrome P450 product complex formation by *N*-hydroxyamphetamine (IV, ○——○), the nitrone (III, ●——●), *N*-hydroxynorbenzphetamine (II, △——△) norbenzphetamine (I, ■——■) in liver microsomes (1.1 μM P450) prepared from control rats. The substrate concentrations were 100 μM. One representative experiment out of six.

was again seen with the amine (I). No complex formation occurred with any of the substrates when sodium dithionite was substituted for NADPH. With *N*-hydroxyamphetamine (IV) and the nitrone (III), incubated under semi-anaerobic conditions (microsomes from phenobarbital-treated rats) the extent of complex formation was decreased by about 70 and 50 per cent respectively.

The level of GSH in isolated hepatocytes was moderately lowered during incubation with norbenzphetamine (I) but the extent of consumption was greater following phenobarbital pretreatment of the rats [7]. As shown in Fig. 4, the hydroxylamine (II), and the nitrone (III), caused the most significant drops in the GSH level, the nitrone (III) being the more effective. Furthermore, the decrease in GSH was enhanced in hepatocytes from phenobarbital-treated rats (Fig. 4B). Simultaneous addition of the nitrone (III) and metyrapone, a known inhibitor of cytochrome P450-linked drug metabolizing enzymes [18], to hepatocytes from phenobarbital-treated rats significantly reduced the effect of III (Fig. 4B). Metyrapone showed no effect on the GSH level in control incubations (data not shown) which was also true for *N*-hydroxyamphetamine (IV) (Fig. 4B). A consistent and constant increase in GSH concentrations prevailing in control incubations, indicated that reduction of GSSG and GSH synthesis [19] were active throughout the incubation period.

Data from simultaneous determinations of GSH and GSSG, using Krebs–albumin buffer as the cell suspension medium, are summarized in Table 1. In the presence of the nitrone (III), more than 50 per cent of the GSH present in the cells was consumed in 1 hr. In this case, a small decrease in GSH concentration was noted also in the control incubation. The amount of GSH present in the supernatant was too small to be detected by the method used. The amount of GSSG in the medium increased to approximately 70 ng min^{-1} × 10^6 $cells^{-1}$ in control incubations, and this amount was not significantly altered in the presence of the nitrone (III).

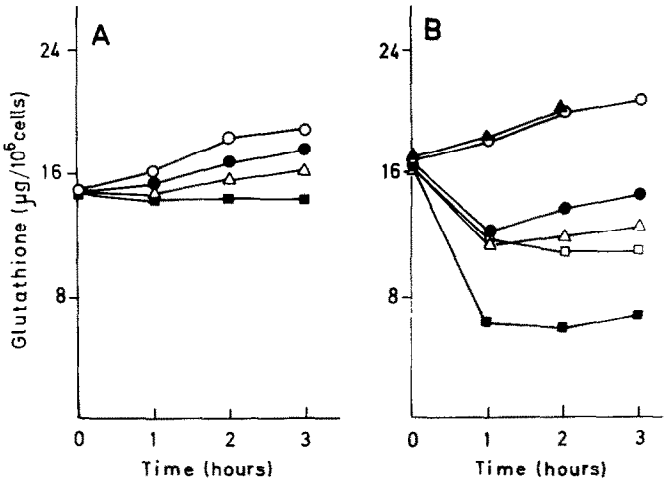


Fig. 4. The effect of substrates I–IV on GSH levels in hepatocytes isolated from control (A) and phenobarbital-treated (B) rats. No substrate (○——○), norbenzphetamine (I, 200 μM, ●——●), *N*-hydroxynorbenzphetamine (II, 200 μM, △——△), the nitrone (III, 200 μM, ■——■), the nitrone + metyrapone (200 μM + 500 μM □——□) and *N*-hydroxyamphetamine (IV, ▲——▲). One representative experiment out of four. For standard deviation cf. Table 1.

Detectable amounts of GSSG were found also within the cells, although they were small compared with those in the medium, and addition of the nitrone did not significantly alter these levels. An immediate excretion of GSSG from the cells to the medium has been reported [20]. Pretreatment of the animals with diethylmaleate, to lower the level of cellular GSH, resulted in a marked increase in the rate of cytochrome P450 complex formation [7].

DISCUSSION

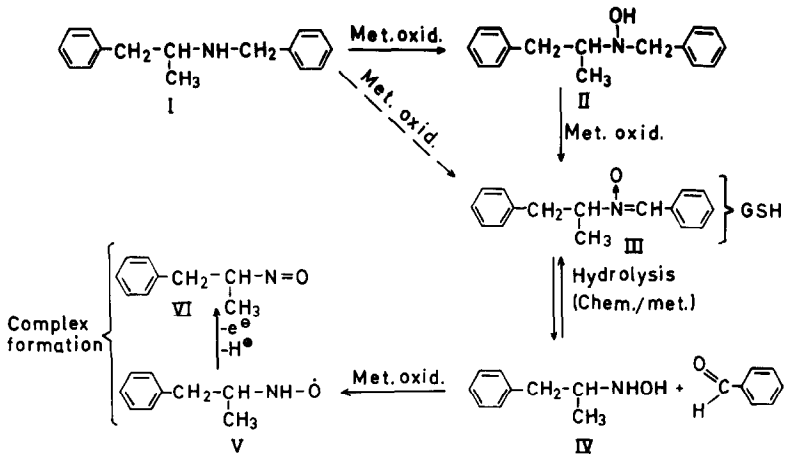
The increased rate of cytochrome P450 product complex formation with liver microsomes and hepatocytes following pretreatment of the animals with phenobarbital, and the inhibitory effect obtained with metyrapone indicate that all the tested compounds (I, II and III) are metabolized by an inducible form of cytochrome P450 prior to complex formation. The enhanced rate and extent of complex formation seen with the hydroxylamine (II) and the nitrone (III) suggest these compounds to be closer to the complex forming metabolite, and in analogy with what has been reported for the primary phenylalkylamines [4,5], *N*-oxidation seems to be a prerequisite for complex formation also with the secondary amine norbenzphetamine (I). Although there is little doubt that *N*-oxidation precedes complex formation, the detailed nature of the complexes formed from amphetamine and its congeners is still obscure and even more so when considering the norbenzphetamine derivatives. As for the primary amines, we have previously suggested the nitroso compounds or the nitroxides as possible ligands [4,5] and Mansuy *et al.* [6] independently presented evidence for the involvement of the nitroso species in complex formation. Very recently, they also established nitrosoalkanes as ligands of iron(II)porphyrins and hemoproteins [21].

Table 1. Levels of reduced (A) and oxidized (B) glutathione during metabolism of the nitrone (III) in isolated hepatocytes prepared from phenobarbital-treated rats*

(A)		GSH (μg/10⁶ cells) 0	1 hr	2 hr
Pellet	no addition	15.5 ± 2.2(4)†	13.0 ± 1.9(4)	11.8 ± 1.7(4)
	200 μM of III	14.3 ± 2.0(4)	6.6 ± 1.0(4)	5.7 ± 0.8(4)
(B)		GSSG (μg/10⁶ cells)* 0	1 hr	2 hr
Pellet	no addition	1.05 ± 0.03(3)	0.85 ± 0.03(3)	0.55 ± 0.03(3)
	200 μM of III	0.9 ± 0.1 (3)	0.70 ± 0.05(3)	0.40 ± 0.05(3)
Supernatant	no addition	0.40 ± 0.07(3)	1.8 ± 0.1(3)	3.1 ± 0.2(3)
	200 μM of III	0.45 ± 0.03(3)	1.9 ± 0.1(3)	3.5 ± 0.3(3)

* Hepatocytes were incubated in 20 ml Krebs–albumin buffer as described in Methods. A 3 ml aliquot was taken out at 1 hr intervals and assayed for GSH and GSSG.

† ± S.E.M. (*n*).



Scheme 1.

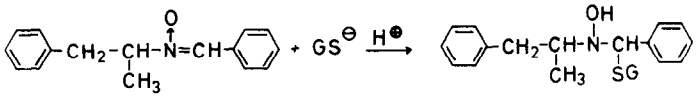
A possibility for norbenzphetamine (I) to be metabolized to amphetamine, which in turn could undergo *N*-oxidation to yield the ligand, was previously taken into consideration [7]. However, this explanation was regarded as less likely in view of the observation that amphetamine failed to produce a complex with cytochrome P450 when incubated with isolated hepatocytes. We have since then shown [5] that the locus for the reaction leading to binding is lipid in nature and that the relatively low activity of amphetamine, found also with microsomal preparations [3,5], is due to its poor distribution into the lipid phase. Norbenzphetamine (I) is considerably more lipophilic than amphetamine and it may then be argued that, if generated from norbenzphetamine (I) at or close to the site for complex formation, amphetamine could readily undergo *N*-oxidation to yield a ligand.

With regard to the chemical properties of nitrones, an alternative and more plausible mechanism can be rationalized for the generation of *N*-hydroxyamphetamine (IV) from norbenzphetamine (I) (Scheme 1). It is known that the condensation of carbonyl compounds with primary hydroxylamines to form nitrones is a rapidly balancing equilibrium [22]. Consequently, the nitrone formed by route of metabolism could undergo partial hydrolysis to benzaldehyde and *N*-hydroxyamphetamine (IV), the latter subsequently being metabolized to the nitroxide (V) or nitroso compound (VI) which acts as the actual ligand. There is evidence in support of this mechanism. Beckett and Gibson [8] have recently established the nitrone (III) to be the major *N*-oxidation product after incubation of I (rabbit liver microsomes) and Kadlubar *et al.* [23] have indicated the chemical instability toward hydrolysis of some nitrones produced after metabolic oxidation of secondary hydroxylamines. Our results show, however, that with the nitrone (III), NADPH-dependent metabolism is a prerequisite for complex formation and the results from the semi-anaerobic experiments imply the involvement of oxygen. Thus, a direct oxidative attack on the nitrone cannot be excluded and further experiments are needed to differentiate between these two possibilities.

The fact that *N*-hydroxyamphetamine (IV) has no effect on the cellular GSH level. while the nitrone (III) decreases the thiol, suggests the nitrone, or a metabolically activated form thereof as the ultimate substrate for GSH. There is some evidence that III reacts with GSH in pure chemical systems at moderately elevated temperatures (unpublished results). A likely reaction is nucleophilic addition [24], (Scheme 2). Such a reaction is also consistent with the unaltered level of GSSG (cf. Table 1). The effects of phenobarbital pretreatment and metyrapone suggest, however, that the reaction is enzymatically catalyzed in the hepatocytes. The induction of GSH *S*-transferases following phenobarbital administration to rats has been established [25].

Thus it appears that the nitrone (III) is a common intermediate in the reactions leading to interaction with GSH and cytochrome P450 complex formation. The latter reaction is in turn facilitated by a lowered cellular level of GSH. Under our incubation conditions the decrease in the GSH level associated with the metabolism of the investigated substrates varied from 20 to 60 per cent (hepatocytes from phenobarbital-treated rats). Most probably, the consumption of GSH was partly compensated for by resynthesis of the thiol, which is quite active under these incubation conditions [cf. 13], and presumably instrumental in protecting the cell from other possible toxic effects of the accumulating nitrone in addition to cytochrome P450 complex formation.

Although the conjugation of GSH with a large number of compounds bearing an electrophilic site has been established [26], there are few examples where the thiol



Scheme 2.

reacts at or close to an activated nitrogen. To our knowledge reactions other than denitrations (i.e. reactions with nitroparaffins [27] and nitrate esters [28]) have not been reported. As metabolic *N*-oxidation is an important route of metabolism for many amines the interaction of various *N*-oxidized compounds with GSH merits further studies.

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REFERENCES

1. J. Werringloer and R. W. Estabrook, *Life Sci.* **13**, 1319 (1973).
2. M. R. Franklin, *Xenobiotica* **4**, 133 (1974).
3. M. R. Franklin, *Molec. Pharmac.* **10**, 975 (1975).
4. J. Jonsson and B. Lindeke, *Acta pharmac. Suec.* **13**, 313 (1976).
5. B. Lindeke, J. Jonsson, G. Hallström and U. Paulsen in *Biological Oxidation of Nitrogen in Organic Molecules* (Ed. J. W. Gorrod.), p. 47. Elsevier, Amsterdam (1978).
6. D. Mansuy, P. Gans, J.-C. Chottard and J.-F.Bartoli, *Eur. J. Biochem.* **76**, 607 (1977).
7. M. Hirata, J. Högberg, H. Thor and S. Orrenius, *Acta pharmac. tox.* **41**, 177 (1977).
8. A. H. Beckett and G. G. Gibson, *J. Pharm. Pharmac.* **29**, 756 (1977).
9. J. C. Schaeffer, A. K. Cho, G. T. Nagami and G. S. Takimoto, *J. Pharm. Sci.* **64**, 1462 (1975).
10. A. H. Beckett, R. T. Coutts and F. A. Ogunbona, *Tetrahedron Lett.* **29**, 4189 (1973).
11. B. Lindeke, A. K. Cho, T. L. Thomas and L. Michelson, *Acta pharmac. Suec.* **10**, 493 (1973).
12. L. Ernster, P. Siekivitz and G. Palade, *J. Cell. Biol.* **15**, 541 (1962).
13. J. Högberg and A. Kristofferson, *Eur. J. Biochem.* **74**, 77 (1977).
14. B. Saville, *Analyst* **83**, 670 (1958).
15. P. J. Hissin, and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
16. P. J. Geiger and S. P. Bessman, *Analyt. Biochem.* **49**, 467 (1972).
17. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
18. K. C. Leibman, *Molec. Pharmac.* **5**, 1 (1969).
19. S. A. Eriksson and B. Mannervik, *Biochim. biophys. Acta* **212**, 518 (1970).
20. J. Högberg, S. Orrenius and P. J. O'Brien, *Eur. J. Biochem.* **59**, 499 (1975).
21. D. Mansuy, P. Battioni, J.-C. Chottard and M. Lange, *J. Am. chem. Soc.* **99**, 6441 (1977).
22. H. Stamm, in *Methodicum Chimicum* (Ed. F. Zymalkowski), Vol. 6, p. 333. Academic Press, New York (1975).
23. F. F. Kadlubar, E. M. Mckee and P. M. Ziegler, *Archs. Biochem. Biophys.* **156**, 46 (1973).
24. E. M. Kosower, in *Glutathion: Metabolism and Function* (Eds I. M. Arias and W. B. Jacoby), Kroc Foundation series **6**, p. 1. Raven Press, New York (1976).
25. W. H. Habig, M. J. Pabst, G. Fleischener, Z. Gatmaitan I. M. Arias and W. B. Jacoby, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3879 (1974).
26. P. L. Grover, in *Drug Metabolism from Microbe to Man* (Eds D. V. Parke and R. L. Smith), p. 105. Taylor and Francis, London (1977).
27. H. G. Bray, J. C. Caygill, S. P. James and P. B. Wood, *Biochem. J.* **90**, 127 (1964).
28. W. H. Habig, J. H. Keen and W. B. Jacoby, *Biochem. biophys. Res. Commun.* **2**, 501 (1975).