cholinesterase inhibitor tetraisopropylpyrophosphoramide had no effect on the activity of the 6.0S enzyme. A similar confirmation of the 5.0S peak as CAT was not possible. The CAT of *Limulus* has been shown to be relatively insensitive to the only readily available specific inhibitors of CAT, the styrylpyridines [1, 20]. Even though a pharmacological confirmation of CAT was not possible, the 5.0S peak represents the majority of the ACh-synthesizing activity present in this tissue. Therefore, the CAT activity is essentially confined within the 5.0S peak, although its homogeneity is uncertain.

The sedimentation profiles of AChE and CAT clearly show the separation of these two enzymes from the solubilized toxin binding proteins. Additionally, αBGT exhibited no detectable effect on Ch uptake. Therefore, the results of this study suggest that αBGT does not interact with AChE, CAT or the site of Ch uptake in the CNS of *Limulus*. These findings are consonant with the specific binding of αBGT to an AChR in *Limulus*.

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REFERENCES

1. P. C. Emson, D. Malthe-Sorenssen, and F. Fonnum, *J. Neurochem.* **22**, 1089 (1974).
2. D. Malthe-Sorenssen and P. C. Emson, *J. Neurochem.* **27**, 341 (1976).
3. C. C. Smith and D. Glick, *Biol. Bull.* **77**, 32 (1939).
4. J. G. Townsel, H. E. Baker and T. T. Gray, *Comp. Biochem. Physiol.* **58**, 29 (1977).
5. W. Schallek, *J. cell. comp. Physiol.* **26**, 15 (1945).
6. J. G. Townsel, H. E. Baker and T. T. Gray, *Neurosci. Abstr.* **2**, (1), 619 (1976).
7. M. A. Maleque, R. F. Newkirk and J. G. Townsel, *Biochem. Pharmac.* **28**, 985 (1979).
8. R. F. Newkirk, M. A. Maleque and J. G. Townsel, *Neuroscience*, **5**, 303 (1980).
9. W. E. Thomas, R. N. Brady and J. G. Townsel, *Archs Biochem. Biophys.* **187**, 53 (1978).
10. W. E. Thomas, R. N. Brady and J. G. Townsel, *Comp. Biochem. Physiol.* **63C**, 199 (1979).
11. D. M. Fambrough, *Physiol. Rev.* **59**, 165 (1979).
12. E. Thomas and J. G. Townsel, *Biochem. biophys. Res. Commun.* **88**, 183 (1979).
13. I. Chao, *Biol. Bull.* **64**, 358 (1933).
14. R. J. Martin and B. N. Ames, *J. biol. Chem.* **236**, 1372 (1961).
15. R. T. Beers and I. W. Sizer, *J. biol. Chem.* **195**, 133 (1952).
16. M. J. Schilesinger and H. Barrett, *J. biol. Chem.* **240**, 4284 (1965).
17. F. Fonnum, *Biochem. J.* **115**, 465 (1969).
18. D. J. Reed, K. Gotto and C. H. Wang, *Analyt. Biochem.* **16**, 59 (1966).
19. J. C. R. Li, in *Statistical Inference*, pp. 51–66. Edwards Brothers, Ann Arbor (1964).
20. H. L. White and C. J. Cavallito, *Biochim. biophys. Acta* **206**, 242 (1970).

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Microsomal peroxidase activities—effect of cumene hydroperoxide on the pyridine nucleotide reduced cytochrome b_5 steady state

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The oxidation of cytochrome b_5 during lipid peroxidation was noted by Bidlack *et al.* [1], who suggested the presence of a hydroperoxide peroxidase in hepatic microsomes. Hrycay and O'Brien [2, 3] and Bidlack and Hochstein [4] characterized the microsomal peroxidase activities, using either NADPH or NADH as reducing equivalents and cumene hydroperoxide as the lipophilic substrate. Most authors now agree that the hepatic mixed function oxygenase serves, at least in part, as the microsomal peroxidase, reducing the lipophilic hydroperoxides to alcohols over cytochrome P-450 [5–9]. Cytochrome b_5, on the other hand, has been implicated only in the transfer of electrons to the NADH mediated peroxidase activity [5–7]. However, immediate oxidation of reduced (NADPH or NADH) cytochrome b_5 occurs upon addition of cumene hydroperoxide [8, 9]. Similarly, using perfused livers and isolated hepatocytes, Sies and Grosskopf [10] reported that the cellular cytochrome b_5 was oxidized upon addition of cumene hydroperoxide.

The central role of cytochrome b_5 in NADH-dependent electron transfer has been examined in cytochrome *c* reductase [11], in stearyl CoA desaturase [12–14], in drug metabolism [15, 16], and in the peroxidase [5–7] activities. However, a more detailed knowledge of the redox state of cytochrome b_5 in the intact microsomal system may clarify the electron transfer through these various pathways. An initial examination of the effect of cumene hydroperoxide on the NADPH and NADH redox state of cytochrome b_5 in microsomes is presented.

Methods

Animal pretreatment. Male Sprague–Dawley rats (*ca.* 250 g) were maintained on Purina Rat Chow. Animals receiving phenobarbital pretreatment were given pheno-

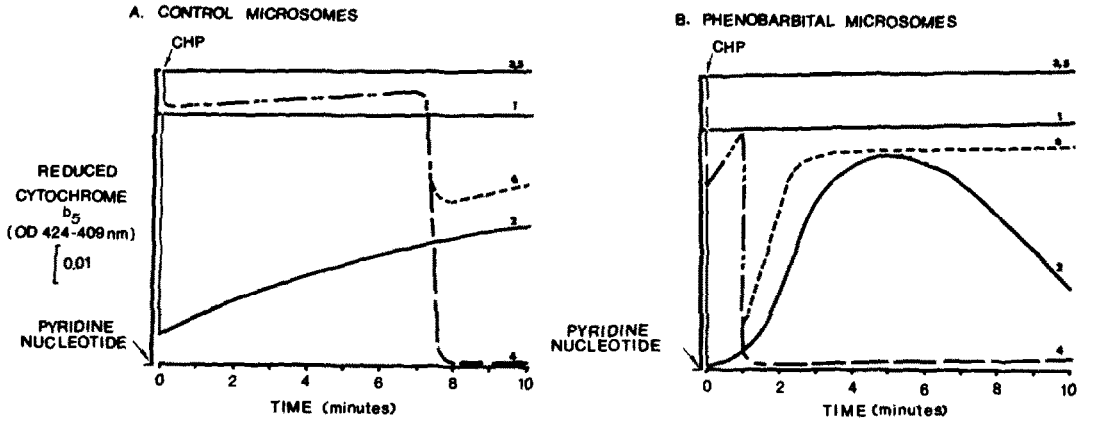


Fig. 1. Cumene hydroperoxide oxidation of pyridine nucleotide reduced cytochrome b_5 in control and phenobarbital-treated microsomes. Microsomes (0.5 mg) were suspended in 1.0 ml Tris (25 mM)–KCl (150 mM) buffer, pH 7.5. The reaction system contained NADPH, NADH or NADPH + NADH (70 μM) and cumene hydroperoxide (100 μM) where indicated. Redox changes in cytochrome b_5 were recorded at 424–409 nm for the pyridine nucleotides alone (curves 1, 3 and 5, respectively) and in the presence of cumene hydroperoxide (curves 2, 4 and 6, respectively). Key: panel A, control microsomes; and panel B, phenobarbital microsomes.

barbital (0.15 g/100 ml) in their drinking water and allowed to drink *ad lib.* for 7–9 days.

Microsome preparation. The microsomal membrane fraction was prepared by the method described previously [17], rinsed, resuspended in Tris (25 mM)–KCl (150 mM) buffer, pH 7.5, and recentrifuged at 105,000 *g* for 30 min. Protein was determined by the method of Lowry *et al.* [18].

Cytochrome b_5 redox analysis. Microsomes (0.5 mg) were suspended in 1 ml of Tris (25 mM)–KCl (150 mM) buffer, pH 7.5. Pyridine nucleotides (NADPH, NADH or both) were added at the concentrations indicated. Cumene hydroperoxide was added, and the resulting spectral changes were recorded for 10 min on an Aminco DW-2 spectrophotometer set in the dual wavelength mode (424 nm maximum and 409 nm minimum). All reactions were carried out at 30°.

Results and discussion

Microsomal cytochrome b_5 was reduced very rapidly by either NADPH or NADH. The steady state reduction of cytochrome b_5 was greater with NADH than with NADPH, but both nucleotides maintained their steady state reduction of cytochrome b_5 in excess of the 10-min reaction period recorded (Fig. 1, panels A and B). Immediate oxidation of the cytochrome occurred upon addition of cumene hydroperoxide (CHP). Initially, the NADPH reduced cytochrome system was oxidized to a much greater extent than the NADH mediated system. However, the NADH reduced cytochrome became totally oxidized, while the NADPH mediated system was slowly re-reduced. In the presence of both pyridine nucleotides, cumene hydroperoxide (CHP) altered the NADH redox state first, oxidizing the cytochrome to the NADPH steady state (Fig. 1A). In microsomes isolated from phenobarbital-pretreated animals (Fig. 1B), the cytochrome b_5 changes occurred at a much faster rate in response to CHP addition. The NADPH reduced cytochrome was oxidized immediately, but with time the cytochrome slowly became re-reduced to the initial NADPH steady state. Initially, the NADH reduced cytochrome was oxidized less by the hydroperoxide, but within 1 min cytochrome b_5 became completely oxidized. In the presence of both NADPH and NADH, the initial oxidation by CHP paralleled the NADH redox changes, but upon further oxidation the cytochrome appeared to be maintained at the NADPH reduced steady state.

Phenobarbital microsomes were used to evaluate the effects of pyridine nucleotide concentration on the cytochrome b_5 redox changes. As might be expected, the reduced state of the cytochrome in the presence of CHP (100 μM) was not maintained at the lower concentrations (50 μM) of NADPH and NADH. But with increased concentrations (100 μM and 150 μM) of the pyridine nucleotides (Fig. 2, panels A and B), the steady state reduction of cytochrome b_5 was maintained for greater periods of time. Interestingly, when NADPH and NADH were combined at various concentrations (Fig. 2C), CHP appeared to oxidize the NADH system first. The NADPH may have been spared, since the reduced cytochrome b_5 was maintained at a steady state level, similar to that produced by NADPH, but for an extended period of time (Fig. 2A). If the NADH was preferentially utilized in the metabolism of the hydroperoxide, then NADPH would not only have been spared, but the concentration of CHP remaining would also be much lower (Fig. 2, line 4 vs line 1). Thus, NADPH could maintain its steady state more effectively for an extended period of time.

The effect of CHP concentration on these cytochrome redox changes was also evaluated (Fig. 3). Again, as might be predicted, the reduced cytochrome was oxidized more completely as the CHP concentration was increased (Fig. 3, panels A and B). In the presence of both NADPH and NADH (Fig. 3C), the NADH steady state was again oxidized to the NADPH steady state, suggesting further that NADH was preferentially used in CHP metabolism.

Jansson and Schenkman [19] have reported redox changes in cytochrome b_5 using a variety of substrates, including aniline, aminopyrine, stearyl CoA and iron–pyrophosphate complexes. These authors, however, used low concentrations of pyridine nucleotides and evaluated the redox changes only in control microsomes. Thus, the observed redox changes of cytochrome b_5 produced by cumene hydroperoxide add to their findings and supplement the consideration of the central role of cytochrome b_5 in electron transfer.

The most unique aspect of the microsomal experiments reported was the effect cumene hydroperoxide had on the cytochrome b_5 redox state in the presence of both pyridine nucleotides, NADPH and NADH. The NADH reduced cytochrome b_5 steady state always underwent a redox change, which was compensated for by a return of the

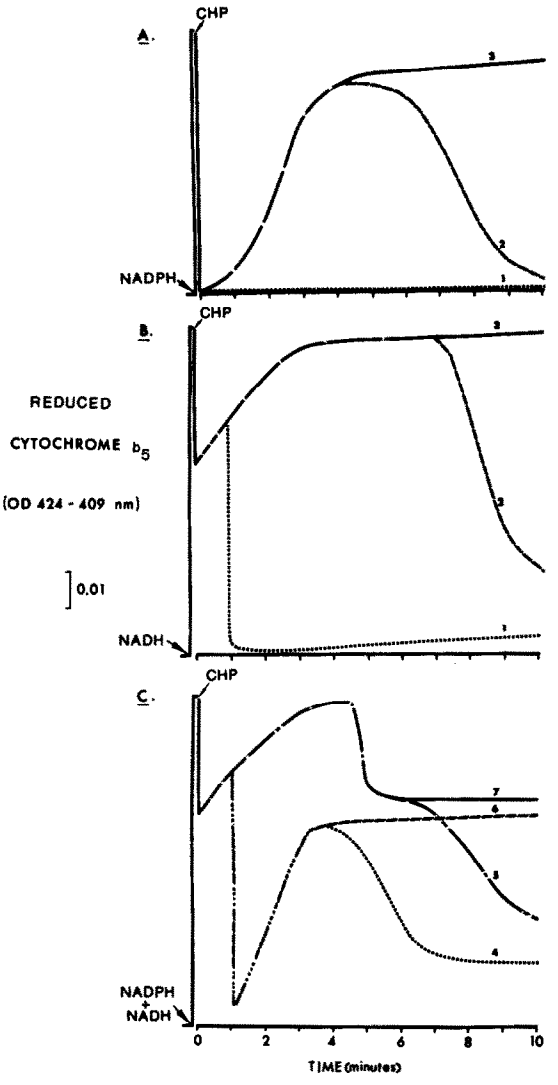


Fig. 2. Effect of pyridine nucleotide concentration on cumene hydroperoxide oxidation of cytochrome b_5. Phenobarbital-treated microsomes (0.5 mg) were suspended in Tris (25 mM)–KCl (150 mM) buffer, pH 7.5. Pyridine nucleotides were added at the concentrations indicated, and the redox changes were initiated by addition of cumene hydroperoxide (100 μM). Panel A, NADPH addition at (1) 50 μM, (2) 100 μM and (3) 200 μM; panel B, NADH addition at (1) 50 μM, (2) 100 μM and (3) 200 μM; and panel C, addition of both NADPH + NADH at (4) 50 μM + 50 μM; (5) 50 μM + 100 μM; (6) 100 μM + 50 μM; and (7) 100 μM + 100 μM, respectively.

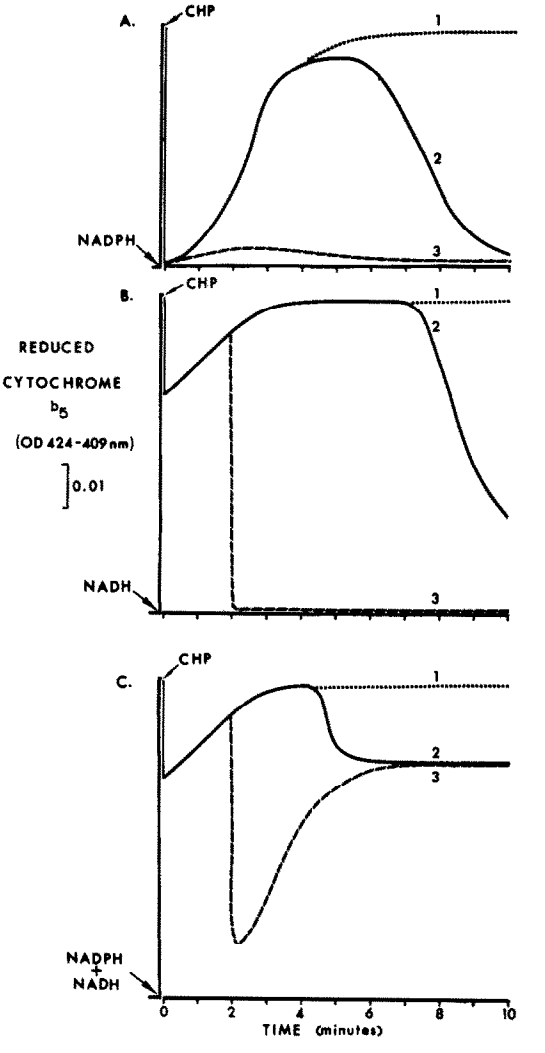
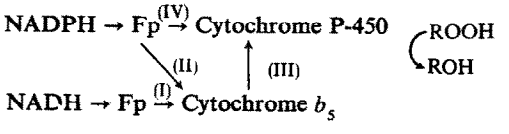


Fig. 3. Effect of cumene hydroperoxide concentration on the pyridine nucleotide reduced cytochrome b_5. Phenobarbital-treated microsomes (0.5 mg) were suspended in Tris (25 mM)–KCl (150 mM) buffer, pH 7.5. Redox changes in pyridine nucleotide (100 μM) reduced cytochrome b_5 were initiated by addition of cumene hydroperoxide at various concentrations: (1) 50 μM, (2) 100 μM and (3) 150 μM. Panel A, NADPH; panel B, NADH; or panel C, NADPH + NADH.

reduced cytochrome to the NADPH steady state. These results suggest a preferential utilization of NADH with the sparing of NADPH.

The microsomal proteins involved with the electron transfer through cytochrome b_5, and which may be involved in the microsomal peroxidase activities, may be characterized by the following simple model:



The rate of transfer of reducing equivalents through each of the numbered pathways may provide a tentative explanation for the observed redox changes. The efficiency of the NADH flavoprotein (I) in reducing cytochrome b_5 is greater than for the NADPH (II) reduction of the cytochrome [19, 20]. To account for the observed redox changes, the electron transfer from cytochrome b_5 to cytochrome P-450, acting as the peroxidase, must be similar to the NADH reduction rate (pathways I $\simeq$ III). Since the preferred pathway for transfer of NADPH reducing equivalents occurs directly to cytochrome P-450 (pathway IV), the oxidation state of cytochrome b_5 may simply reflect the redox state of cytochrome P-450 reductase. If the NADPH mediated rate of CHP metabolism exceeded the rate of electron transfer to cytochrome b_5, then electron transfer over pathway II would be diminished and cytochrome b_5 would become oxidized.

Indeed, the preferential utilization of NADH would be expected in part since the rate of the NADH microsomal peroxidase activity exceeds the NADPH mediated peroxidase activity [2–4, 8]. Then, as the CHP concentration decreases (*ca.* 80 per cent CHP reduced in 3 min), the remaining NADPH can re-reduce the cytochrome b_5 more efficiently to its reduced state.

In addition, Werringloer and Estabrook [21] have reported that the oxidation of NADH was increased in the presence of NADPH, both in the presence and absence of substrate. The oxidation appeared to be dependent on the concentration of NADPH as well. This effect would also increase the conversion of the NADH maintained reduced cytochrome b_5 steady state to the NADPH reduced cytochrome steady state in the presence of CHP.

Thus, cytochrome b_5, reduced by either NADPH or NADH, was oxidized in the presence of CHP. NADH maintained the reduced steady state of cytochrome b_5 more efficiently than did NADPH. Yet, in the presence of both pyridine nucleotides, the hydroperoxide initially oxidized the cytochrome to the NADH mediated steady state but was then oxidized further to the NADPH reduced steady state. From these redox changes, we conclude that NADH was used either preferentially or at a faster rate, sparing the NADPH which was then available to re-reduce cytochrome b_5 to the NADPH steady state.

More detailed experiments are in progress, quantitating each of the pyridine nucleotides and the CHP with time. These experiments should clarify the exact causes of the redox changes observed and may improve our understanding of the central role played by cytochrome b_5 in the microsomal electron transfer reactions, especially to the microsomal peroxidase activities.

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REFERENCES

1. W. R. Bidlack, R. T. Okita and P. Hochstein, *Biochem. biophys. Res. Commun.* **53**, 459 (1973).
2. E. G. Hrycay and P. J. O'Brien, *Archs Biochem. Biophys.* **157**, 7 (1973).
3. E. G. Hrycay and P. J. O'Brien, *Archs. Biochem. Biophys.* **160**, 230 (1974).
4. W. R. Bidlack and P. Hochstein, *Life Sci.* **14**, 2003 (1974).
5. E. G. Hrycay and R. A. Prough, *Archs Biochem. Biophys.* **165**, 331 (1974).
6. E. G. Hrycay and R. W. Estabrook, *Biochem. biophys. Res. Commun.* **60**, 771 (1974).
7. E. G. Hrycay, H. G. Jonen, A. Y. H. Lu and W. Levin, *Archs Biochem. Biophys.* **166**, 145 (1975).
8. W. R. Bidlack, *Proc. west. Pharmac. Soc.* **21**, 329 (1978).
9. W. R. Bidlack, *Fedn Proc.* **37**, 1544 (1978).
10. H. Sies and M. Grosskopf, *Eur. J. Biochem.* **57**, 513 (1975).
11. M. J. Rogers and P. Strittmatter, *J. biol. Chem.* **248**, 800 (1973).
12. N. Oshino, Y. Imai and R. Sato, *J. Biochem., Tokyo* **69**, 155 (1971).
13. N. Oshino and R. Sato, *J. Biochem., Tokyo* **69**, 169 (1971).
14. N. Oshino and T. Omura, *Archs Biochem. Biophys.* **157**, 395 (1973).
15. H. A. Sasame, S. S. Thorgeirsson, J. R. Mitchell and J. R. Gillette, *Adv. exp. Med. Biol.* **58**, 435 (1974).
16. S. B. West and A. Y. H. Lu, *Archs Biochem. Biophys.* **182**, 369 (1977).
17. D. G. Wolff and W. R. Bidlack, *Biochem. biophys. Res. Commun.* **73**, 850 (1976).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. I. Jansson and J. B. Schenkman, *Archs Biochem. Biophys.* **185**, 251 (1978).
20. J. Modirzadeh and H. Kamin, *Biochim. biophys. Acta* **99**, 205 (1965).
21. J. Werringloer and R. W. Estabrook, *Biochem. biophys. Res. Commun.* **71**, 834 (1976).

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Effects of morphine and methadone on the isolated perfused rat brain

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The variable results obtained previously [1–4] in studies in rats of the effects of the administration of morphine on the intermediary metabolites and energy reserves of brain tissue may have resulted from differences in the techniques of sampling brain tissue for analysis. We have studied the direct effects of morphine and also methadone on the rat cerebral cortex, using an isolated perfused rat brain preparation [5]. With this intact brain preparation, there is no interference from other organs, no anesthetic agent is used, blood flow and blood gases are controlled, and reproducible samples of cerebral cortex are very rapidly frozen for analysis. We found that perfusion of this isolated brain with fluid containing morphine (50 μg/ml) caused significant decreases in the concentrations of ATP and creatine phosphate and a significant increase in lactate. In contrast, brains perfused with methadone (50 μg/ml) showed very little change in brain metabolites, only a small decrease in ATP, and an increase in lactate.

Methods

Preparation and perfusion of isolated brain. Unanesthetized male Sprague–Dawley rats (about 250 g) were adequately anesthetized for surgery by deep hypothermia (rectal temperature 16–18°) induced as described previously [6]. The isolated brain preparation, consisting of the detached skull and its contents, was made from the hypothermic rat without lapse of circulation and was perfused through the internal carotid arteries as described previously [5]. The perfusion fluid [7] contained the perfluoro compound FC-80 (3M Co., St. Paul, MN) as an erythrocyte substitute which was dispersed ultrasonically in an 8% (w/v) solution of bovine albumin (fraction V powder, Sigma