THE ROLE OF NADPH-CYTOCHROME b_5 REDUCTASE IN MICROSOMAL LIPID PEROXIDATION

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SUMMARY: Spectrophotometric changes in the extent of NADPH, but not NADH, reduction of microsomal cytochrome b_5 are correlated with the utilization of oxygen and the accumulation of lipid peroxidation products. The results suggest that NADPH-cytochrome b_5 reductase (NADPH-cytochrome c reductase) participates in the reduction of obligatory ferric chelates to their ferrous form prior to the initiation of lipid peroxidation. Further, an increased oxidation of cytochrome b_5 observed only in the presence of peroxidation products implicates a peroxidase activity associated with b_5 in the microsomal electron transport chain.

The specific mechanisms of the enzymatic lipid peroxidizing system, first described by Hochstein and Ernster (1,2,3) in rat liver microsomes, have remained obscure. The system requires NADPH, but not NADH, iron in either ferric or ferrous forms, the presence of certain chelators including pyrophosphate, ADP, ATP, etc., and oxygen. Orrenius, et al. (4) have described an interaction between enzymatic lipid peroxidation and NADPH-dependent drug hydroxylation which is evidenced by the inhibition of peroxidation during the hydroxylation of various drugs. Their experiments suggest that the two processes share a common NADPH oxidizing system.

Recently, Pederson and Aust (5) have demonstrated that purified cytochrome c reductase may induce a peroxidation of admixed lipids through the generation of superoxide anions. However, the relevance of such a reaction is obscured by the fact that it requires EDTA. This agent is a known inhibitor of lipid peroxidation in the intact microsomal system.

The participation of cytochrome b_5 in drug hydroxylation, described by Estabrook and his coworkers (7,8), has not only provided a clearer insight into the mechanisms of drug metabolism, but also suggests the possible involvement of this cytochrome in enzymatic lipid peroxidation. The experi-

ments reported in this paper, in fact, implicate a dual role for the microsomal cytochrome b_5 electron transport chain in NADPH-dependent peroxidation reactions.

METHODS: Rat liver microsomes were prepared daily by minor modifications of the procedure of Ernster, et al (9). The microsomal pellet from fasted animals was collected from a 28,000 g supernatant fraction by centrifugation at 105,000 g for 60 minutes. The surface of the pellet was thoroughly rinsed and the microsomes resuspended in TRIS (25.0 mM)-KCL (150.0 mM) buffer, pH 7.4. Incubations were carried out in this buffer at 22-25° C. Oxygen was measured polarographically with a Clark-type electrode. Lipid peroxidation products were determined as thiobarbituric acid reacting substances (TBRS). For these determinations, the reactions were stopped at appropriate times with 1.0 ml of 20% TCA. The mixtures were centrifuged for 10 minutes at 1,000 g. One ml of the resulting supernatant was removed and added to 1.0 ml of aqueous thiobarbituric acid (0.75%). The tubes were heated in a boiling water bath for 20 minutes, cooled to room temperature, and the optical densities at 532 nm were recorded. Cytochrome b_5 was measured with an Aminco dual wavelength spectrophotometer at 557 nm with 567 nm as the reference wavelength.

RESULTS: Figure 1 is a composite recording which shows the reduction of microsomal b_5 subsequent to the addition of reduced pyridine nucleotides, and the changes caused by the addition of the ferric chelate, ADP-Fe^{+3}. The lower two curves (E and F) indicate the actual extent of the reduction of b_5, from the baseline, after the addition of pyridine nucleotides, while the upper curves (A,B,C and D) show only the optical densities without the base line reference. However, the extent of the reduction observed in these latter instances was the same as that shown in curves E and F, and in all cases the scale is constant.

Addition of NADPH to the microsomes causes a prompt reduction of b_5 to a steady state level that is maintained during the 3 minute recording period

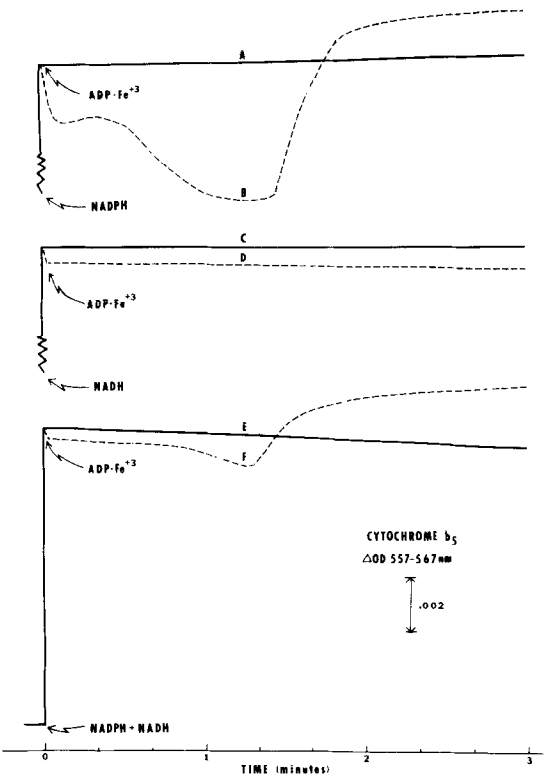


Figure 1. The effect of ADP-Fe^{+3} on the steady-state of pyridine nucleotide reduced microsomal cytochrome b_5. A final volume of 3.0 ml. Tris (25 mM)-KCL (150 mM) pH 7.4 buffer contained: Microsomes, 3.0 mg.; NADPH and/or NADH 0.3 mM; ADP, 2.5 mM; and $FeCl_3$, 25 mM.

(curve A). Now, the addition of the ferric chelate (curve B) results in an immediate and rapid oxidation of b_5, a small but reproducible re-reduction, and then a more prolonged period of oxidation. This latter period is terminated after about one minute. At this point b_5 becomes reduced to a level higher than the initial steady-state had allowed. When NADH is used as the reducing nucleotide, b_5 is again reduced to the same level as when NADPH was employed (curve C). However, the addition of the ferric chelate, in this instance, causes only a small shift in the steady-state to the oxidized side (curve D). The simultaneous addition of both NADH and NADPH (curve E) causes a reduction of b_5 to the same level as with either nucleotide alone. When both NADH and NADPH are present, the addition of ferric chelate does

TABLE 1: OXYGEN UPTAKE AND TBRS FORMATION IN ENZYMATIC LIPID PEROXIDATION

	O_2 (n moles/min)	TBRS (OD_{532} at 3')
1. NADPH	6	.08
2. NADPH + ADP-Fe^{+3}	246	.94
3. NADH	16	.06
4. NADH + ADP-Fe^{+3}	19	.09
5. NADPH + NADH	23	.11
6. NADPH + NADH + ADP-Fe^{+3}	260	.67
7. NADPH + ADP	23	.10
8. NADPH + Fe^{+3}	12	.18

The one ml. reaction system of Tris (25 mM)-KCL (150 mM) buffer, pH 7.4 contained: microsomes, 1.0 mg protein; pyridine nucleotides, 0.3 mM; ADP, 2.5 mM; and $FeCl_3$, 25 uM where indicated.

not result in the initial rapid oxidation of the b_5 (curve F). However, a second oxidation is noted after about 40 seconds prior to an upward deflection in the recording, and the attainment of the higher reduced steady-state level characterized by that observed with NADPH alone.

Concommitantly with the changes in the reduction of cytochrome b_5 described in Figure 1, O_2 consumption and the formation of thiobarbituric acid reacting substances (TBRS) was measured. Table I shows that ferric chelates stimulate O_2 uptake and the accumulation of TBRS only in the presence of NADPH (exp. 1,2 and 5-8). NADH was not active in this respect (exp. 3,4). In the presence of both NADH and NADPH good rates of O_2 uptake were observed after the addition of the ferric chelate, although the formation of TBRS was consistently less (exp. 6) than that observed with NADPH alone (exp. 2). The nature of this anomolous finding is under investigation.

In Figure 2 the kinetics of cytochrome b_5 oxidation-reduction is compared directly with O_2 uptake and the accumulation of TBRS in the microsomal

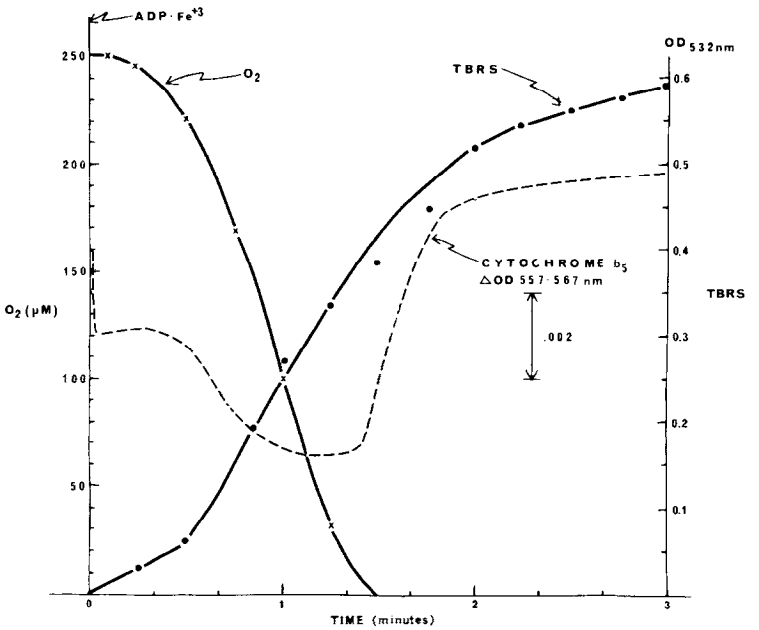
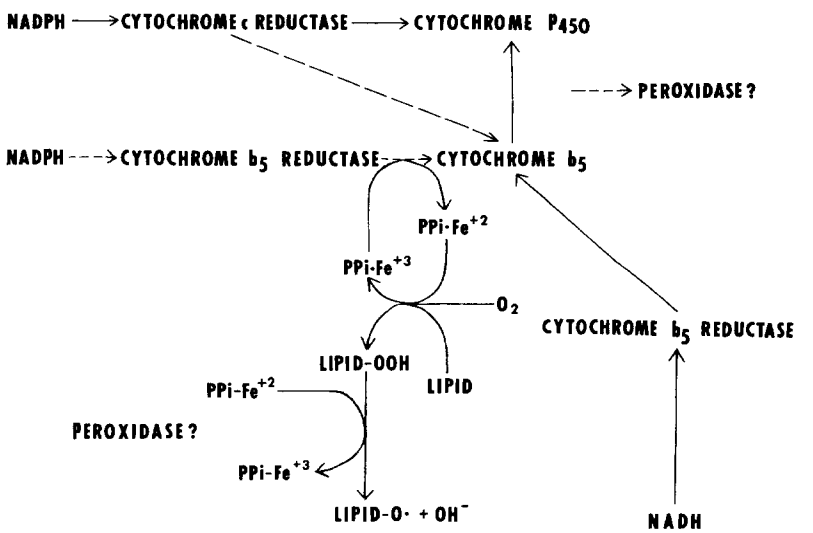


Figure 2. The simultaneous effects of ADP-Fe^{+3} on oxygen uptake, TBRS formation, and the steady-state of NADPH reduced cytochrome b_5. The reaction conditions were the same as those indicated in Figure 1.

system. In all cases the ferric chelate was added at zero time. Of major interest is the initial oxidation of b_5 prior to detectable O_2 utilization. In the period from 5 to 20 seconds, the partial re-reduction of b_5 is accompanied by the initiation of O_2 utilization and the formation of TBRS. The period of the second oxidation of b_5 is simultaneous with the maximum rate of O_2 uptake and TBRS formation. As the reaction system goes anaerobic (ca. 80-100 seconds) the steady-state reduction of cytochrome b_5 is shifted to a level higher than that observed in the aerobic system before the addition of ferric chelate. At this point the accumulation of TBRS is almost maximal, although a small increase is still recorded after the system is completely anaerobic.

DISCUSSION: We visualize the results of these experiments and the interaction of the enzymatic lipid peroxidizing system with the microsomal electron transport chain involved in drug hydroxylation as shown in the following scheme:



Cytochrome b_5 is known to be reduced by NADH and NADPH. The NADH specific reductase has been well-described by Strittmatter and coworkers (10,11,12). But NADPH cytochrome b_5 reductase has only been vaguely mentioned in the literature (12) and is most generally referred to as NADPH-cytochrome c reductase (7,13). Recently, Sato and coworkers (14) have shown that NADPH-cytochrome c reductase prepared by detergent solubilization may indeed reduce cytochrome b_5.

In any case, in our experiments the reduction of b_5 in the presence of both NADPH and NADH does not exceed that obtained with either nucleotide alone. This and the presence of two cytochrome b_5 reductases suggests either, that both enzymes utilize the same b_5, or that separate pools of cytochrome b_5 exist which are in rapid equilibrium.

Since NADH was shown not to support lipid peroxidation, and the NADH reduced cytochrome b_5 was not oxidized by the ferric chelate, the peroxidizing system must occur prior to cytochrome b_5 and most likely off the NADPH cytochrome b_5 reductase. Whether or not this enzyme is the same or different from the NADPH cytochrome c reductase is yet to be established. In either case, the data presented indicates that the initial oxidation of cytochrome b_5 may simply reflect the reduction of the ferric chelate via the NADPH-

cytochrome b_5 reductase. Activation of the oxygen, formation of the lipid peroxides, and their turnover may occur by a simple Fenton's type reaction (15) as described in the scheme.

It is of special interest that the second oxidation of cytochrome b_5 was observed only during active peroxidation and, although not described in this paper, it could be interrupted by EDTA (6). These results suggest the occurrence of an electron drain involving peroxides, the iron chelate, and a peroxidase activity that may occur either, after, or directly off, cytochrome b_5. Hrycay and O'Brien (16,17) have indicated that cytochrome P-450 may have peroxidase activity and the existence of such peroxidase activity in the lipid peroxidizing system has been previously hypothesized by Bidlack and Tappel (18).

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