ADP-ACTIVATED LIPID PEROXIDATION COUPLED TO THE TPNH OXIDASE SYSTEM OF MICROSOMES

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The presence of lipid peroxides, detected by the formation of malonaldehyde (MA), has been observed in tissues isolated from vitamin E-deficient animals (Zalkin and Tappel, 1960). Lipid peroxidation has also been found to occur in isolated subcellular fractions when these were incubated aerobically in the presence of hematin compounds (Tappel and Zalkin, 1960), ferrous ions (Ottolenghi, 1959; Hunter et al., 1963), and ascorbate (Ottolenghi, 1959; Thiele and Huff, 1960).

In this paper, we wish to report the occurrence of a TPNH-induced peroxidation of lipids in rat-liver microsomes. The reaction requires activation by ADP or other pyrophosphates, and appears to be coupled to the TPNH oxidase system of the microsomes. The latter system was first described by Gilette et al. (1956) in connection with studies of TPNH-linked hydroxylations.

Some properties of the TPNH-induced lipid peroxidation are described below and compared with those of the non-enzymically induced lipid peroxidation which occurs in the presence of ascorbate.

Methods.- Microsomes from 0.25 M sucrose homogenates of rat liver were prepared as described by Ernster et al. (1962). In order to remove the bulk of the sucrose (which interferes with the determination of malonaldehyde) the microsomes were washed once with 0.15 M KCl. The pellets were finally suspended in 0.15 M KCl to contain 10 to 30 mg protein/ml. Incubations were made in a tris (0.025 M) - KCl

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(0.15 M) medium, pH 7.5, at 22-25°C. The composition of the reaction mixture is further specified in the legends to the tables and figures. Oxygen consumption was measured polarographically using a stationary Pt electrode and a rotating cuvette.

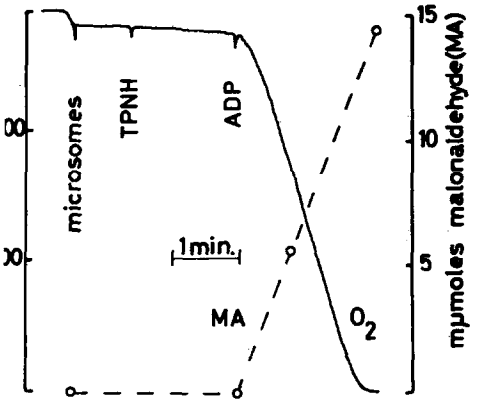


Fig. 1. TPNH and ADP dependent O_2 uptake and MA formation. Additions when indicated were: microsomes containing 6 mg protein, TPNH, 0.3 mM, ADP, 1.0 mM. Final vol. was 1.2 ml.

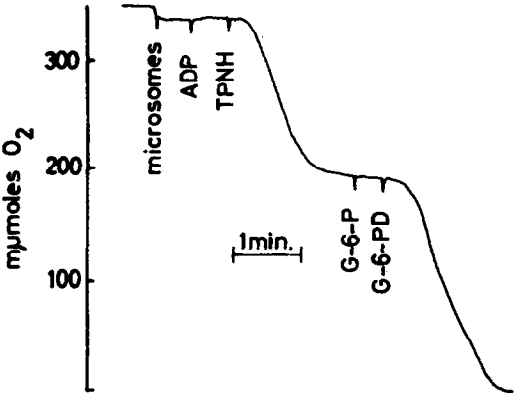


Fig. 2. O_2 uptake with limiting amounts of TPNH and with a TPNH generating system. Additions were: microsomes, 6 mg protein, ADP, 1.0 mM, TPNH, 0.01 mM, G-6-P, 10 mM, G-6-P-Dehydrogenase (G-6-P-D), 0.1 mg Nicotinamide, 40 mM was also present. Final Volume, 1.5 ml.

TPNH disappearance was followed in an Eppendorf fluorimeter as described by Estabrook and Maitra (1962). Malonaldehyde was determined by the thiobarbituric acid reaction (Bernheim et al., 1947); an extinction coefficient of $A^{1\ cm}_{535\ m\mu} = 1.56 \times 10^5$ was used (Sinnhuber et al., 1958).

Results.- When a suspension of rat liver microsomes was incubated with TPNH, only a slow rate of O_2 uptake was observed, and no measurable amount of MA was formed (Fig. 1). However, addition of ADP resulted in a rapid O_2 consumption, paralleled by a formation of MA. The molar ratio of O_2 consumed to MA formed was about 20.

Both the O_2 consumption and the formation of MA were dependent on the presence of TPNH. When a limited amount of TPNH was used (Fig. 2), O_2 consumption and MA formation (not shown) ceased after a while, and could be re-initiated by the addition of glucose-6-phosphate and glucose-6-phosphate dehydrogenase.

Data relating to the specificity of TPNH and ADP are summarized in Table I. Replacement of TPNH by DPNH resulted in only negligible O_2 uptake and MA formation. The ADP requirement was not specific, but at the concentration where ADP gave maxi: al effect, ca. 4 mM, other nucleoside diphosphates, as well as ATP, were less acti: Inorganic pyrophosphate did not markedly activate the reaction at 4 mM, but did so at lower concentrations. No activation of O_2 consumption or MA formation was obser: with AMP or inorganic ortho-phosphate in the concentration range of 0.01 to 10 mM.

Table I. TPNH Requirement: the effect of various pyrophosphates. Microsomes containing 6 mg protein were used. Final volume of reaction mixture was 1.5 ml.

Additions	O_2	MA	O_2:MA
	mμmoles/min./mg protein		
ADP, 4 mM	0	0	--
DPNH, 0.3 mM, ADP, 4 mM	4.4	0.21	20.9
TPNH, 0.3 mM, ADP, 4 mM	94.1	4.34	21.7
" ADP, 3 mM	84.0	4.13	20.3
" ADP, 2 mM	62.1	3.10	19.8
" ADP, 1 mM	28.9	1.44	20.0
" ATP, 4 mM	36.4	1.69	21.5
" GDP, 4 mM	39.8	1.98	20.1
" UDP, 4 mM	14.2	0.63	22.5
" CDP, 4 mM	32.0	1.57	20.4
" IDP, 4 mM	27.6	1.26	21.9
" PP, 4 mM	5.4	0.24	22.5
" PP, 1 mM	15.9	0.79	20.1
" PP, 0.1 mM	39.6	1.95	20.3
" PP, 0.01 mM	9.0	0.43	20.9
" PP, 0.001 mM	0	0	--

Table II presents data concerning the stoichiometry of the reaction. In the absence of ADP, approximately one mole of O_2 was consumed per mole of TPNH disappearing; the formation of MA was insignificant. Four mM ADP stimulated the TPNH

Table II. Stoichiometry of TPNH-induced linid peroxidation. Additions were: TPNH, 0.3 mM, ADP, 4.0 mM. Microsomes containing 12 mg protein were used to measure TPNH disappearance and the final volume of the reaction mixture was 3.0 ml. Microsomes containing 6 mg protein were used to measure O_2 consumption and MA formation and the volume was 1.5 ml.

	TPNH disapp.	O_2 consum.	MA formed
	mμmoles/min./mg protein		
- ADP	1.22	1.30	<0.1
+ ADP	4.03	87.35	4.68

disappearance 3 to 4-fold and the O_2 uptake about 60-fold; when 1 mM ADP was used, the stimulation of the O_2 uptake was only about 15 fold whereas that of the TPNH disappearance remained 3 to 4 fold. The amount of MA formed, as previously indicated, was about 1/20th the amount of O_2 consumed, and this value was constant regardless of the extent of stimulation of the O_2 uptake (cf. Table I).

The following compounds were found to inhibit both O_2 uptake and MA formation in the TPNH- and ADP-supplemented system (Fig. 3): a) the antioxidants, diphenyl-phenelenediamine (DPPD), and 2-(3-hydroxy-3-methyl-carboxypentyl)-3,5,6-trimethyl-benzoquinone (αTM); the latter compound is an active metabolite of α-tocopherol (Simon et al., 1956); b) the known inhibitor of TPNH-dependent microsomal hydroxylations, β-diethylaminoethyl-diphenylpropylacetate (SKF 525A); c) ethylenediaminetetra-acetate (EDTA); and d) p-chloromercuribenzoate (PCMB). All of these compounds inhibited TPNH disappearance as well, yet, with the exception of PCMB, only that portion

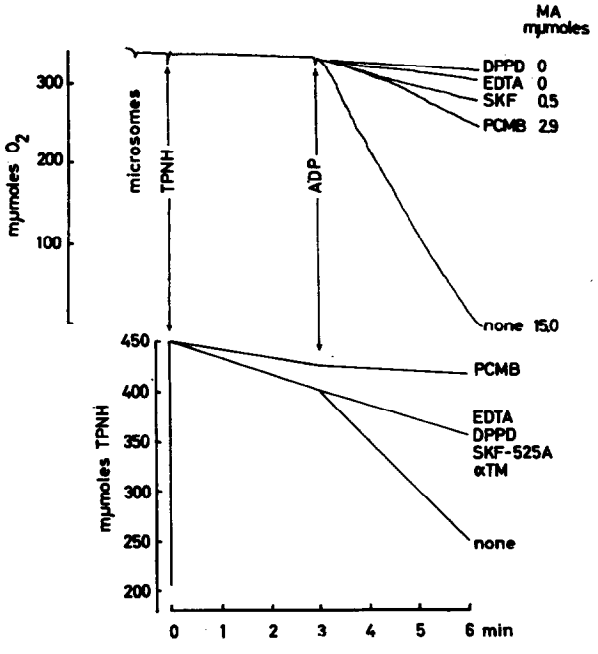


Fig. 3. The effect of various inhibitors on O_2 uptake, MA formation and TPNH disappearance. Additions when indicated were: microsomes containing 4 mg protein in O_2 uptake experiment and microsomes containing 12 mg protein in TPNH disappearance experiment, TPNH, 0.3 mM, ADP, 1.0 mM. Inhibitors were added prior to the addition of the microsomes: DPPD, 0.1 γ, αTM 2 γ, EDTA, 0.01 mM, SKF-525A, 1.0 mM, and PCMB, 0.16 mM. Final volume was 1.5 ml in O_2 uptake experiment and 3.0 ml in TPNH disappearance experiment.

Table III. The effect of various inhibitors on TPNH- and ascorbate-induced lipid peroxidation. Microsomes containing 6 mg protein were used. ADP, 4.0 mM, TPNH, 0.3 mM or ascorbate, 1.0 mM were added in a final volume of 1.5 ml.

Additions	MA formed/min./mg protein	
	TPNH	Ascorbate
None	5.71	3.87
DPPD, 0.1 γ	0.12	0
αTM, 5.0 γ	0.14	0.12
EDTA, 0.01 mM	0.17	0.10
SKF-525A, 0.1 mM	3.21	2.33
PCMB, 0.16 mM	0.75	4.45
heat (5-100°)	0	3.50
" -ADP	--	0.41
-ADP	--	0.30

of the TPNH disappearance resulting from the addition of ADP. Inhibitory effects on the reactions studied were also observed with glutathione (1 mM) and with sodium deoxycholate (0.1 %). Cyanide (1 mM) and crystalline catalase had little or no effec

In Table III are data concerning MA formation induced by ascorbate and by TPNH In both systems, ADP activated the formation of MA, and EDTA, SKF, DPPD and αTM were inhibitory. However, in contrast to the TPNH system, the ascorbate system was insensitive to PCMB and to heat denaturation of the microsomes. It should be noted tha ADP activation of the ascorbate-induced O_2 uptake and MA formation also occurred in heat-denatured microsomes, showing that this effect in all likelihood is non-enzymic ADP-activation of the ascorbate-induced lipid peroxidation was also observed in fres as well as heat-denatured mitochondria. However, no TPNH-induced lipid peroxidation could be demonstrated in mitochondria (Fig. 4).

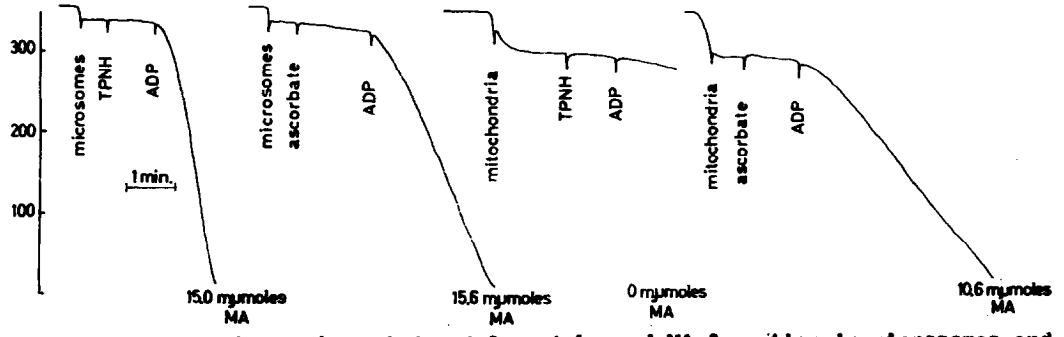
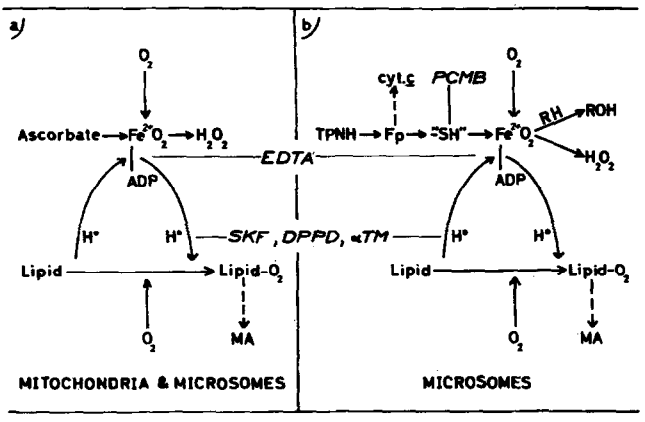


Fig. 4. TPNH- and ascorbate-induced O_2 uptake and MA formation in microsomes and mitochondria. Additions were: microsomes containing 6 mg protein or mitochondria containing 4 mg protein, TPNH, 0.3 mM, ascorbate, 1.0 mM, ADP, 4.0 mM. The final volume was 1.5 ml. Mitochondria were prepared by the method of Ernster and Löw (1955) from a portion of the homogenate used to prepare microsomes. The "fluffy layer" was thoroughly decanted in order to minimize microsomal contamination.

TPNH- and ADP-dependent O_2 uptake and MA formation were observed with microsomes from kidney and brain, but not with those of skeletal muscle or ascites tumor cells. This distribution appeared to parallel that of TPNH cytochrome c reductase.

Discussion.- From the results reported above it is concluded that microsomes catalyze an ADP-activated peroxidation of lipids, coupled to the TPNH oxidase system. The peroxidation of lipids is indicated by the formation of malonaldehyde, which is generally recognized as a split product of certain lipid peroxides. Furthermore, the oxygen consumption observed in the presence of ADP greatly exceeded the disappearance of TPNH, and must therefore be accounted for by the oxidation of microsomal material. Of conceivable microsomal components only lipids are present in such quantities as to account for the amounts of oxygen consumed. Finally, the peroxidation of lipids is strongly implicated by the inhibitions observed with the antioxidants, DPPD and αTM - these being known as potent inhibitors of lipid peroxidation.

Mechanistically, the TPNH-linked, enzymically-induced lipid peroxidation appears to be essentially analogous to that induced by ascorbate. Schemes a and b below summarize our tentative interpretation of the reaction mechanisms involved in the two processes. Scheme a involves a non-enzymic, iron-catalyzed oxidation of ascorbate, with $Fe^{2+}O_2$ as an intermediate and H_2O_2 as the reaction product (cf. Mason, 1957). Scheme b describes the components of the TPNH oxidase system as suggested by current information, i.e., a flavoprotein presumably identical with Horecker's (1950) TPNH-cytochrome c reductase (cf. Krisch and Staudinger, 1961, Phillips and Langdon, 1962), a sulfhydryl intermediate, and Fe^{++}. In the presence of TPNH and O_2, $Fe^{2+}O_2$ is probably



formed (cf. Mason, 1957), with the subsequent formation of either H_2O_2 or, when a hydroxyl acceptor is available, ROH (cf. Brodie, 1962). We visualize that, in both cases, the lipid peroxidation is catalyzed by an ADP (or other pyrophosphate) chelate of FeO_2. The catalysis is thought to consist of the shuttling of a hydrogen atom from and to the lipid undergoing peroxidation, analogous to the mechanism proposed for plant lipoxidase (Bergström and Holman, 1948). In forthcoming papers we shall present other details of the reaction mechanism, as well as data bearing on the relationship between lipid peroxidation, microsomal structure, and the activity of various microsomal enzymes.

While this work was in progress, Beloff-Chain et al. (1963) have shown that ADP, and other nucleoside diphosphates, enhance the TPNH oxidase activity of rat liver microsomes. Concomitant lipid peroxidation was not reported.

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