LIPID ALTERATIONS OCCURRING IN MICROSOMES DURING THE ENZYMIC OXIDATION OF TPNH

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Hochstein and Ernster (1963) have recently reported that when microsomes were incubated in the presence of TPNH, the addition of ADP resulted in a marked increase in TPNH oxidation and oxygen uptake. In addition, there was formation of a chromogen reacting with thiobarbituric acid (TBA) and designated as malonaldehyde. The high oxygen uptake, in excess of that required for TPNH oxidation, and the formation of chromogen were attributed to peroxidation of microsomal lipids.

We have analyzed the lipid from rat liver microsomes which were incubated at hyperbaric pressures of oxygen in the presence (Experimental) and absence (Control) of TPNH and ADP. Marked changes in the chromatographic behavior and the chemical characteristics were observed only in the lipids extracted from the experimental system.

Materials: Washed microsomes were prepared by differential centrifugation of rat liver homogenates in 0.15 M phosphate buffer, pH 7.5, and stored in the pellet form at -20°C. Just before use, the microsomes were suspended in 0.1 M Tris, pH 7.5, so that 0.1 ml of the suspension contained the quantity of microsomes from 100 mg of liver.

Results and Discussion: It was previously observed (McCay et al., 1960) that oxygen pressure above one atmosphere activates gulonolactone oxidase, another microsomal enzyme also producing a TBA-reacting chromogen which is dependent on the enzymic oxidation of gulonolactone. It was of interest, therefore, to investigate the effect of hyperbaric oxygen pressures on the TPNH-ADP-dependent TBA chromogen formation in microsomes

reported by Hochstein and Ernster. These results are summarized in Table I. The rate of chromogen formation is increased by a factor of

TABLE I

The Effect of Oxygen Pressure on the Rate of TBA Chromogen Formation. Incubation systems: Control--0.1 ml microsomes and 0.9 ml 0.1 M Tris, pH 7.5. Experimental--0.1 ml microsomes, 0.3 μmoles TPNH, 4 μmoles ADP and 0.9 ml 0.1 M Tris, pH 7.5. Chromogen formation determined by method of Ottolenghi (1959).

Oxygen Pressure (Atm)	Incub. Time (Min)	OD_{532}/ml Incub.	
		Experimental	Control
0.2	7	0.94	----
0.2	15	1.69	0.06
0.2	30	2.22	0.08
5.0	7	2.16	----
5.0	15	3.92	0.16
5.0	30	5.53	0.53

two at five atmospheres of O_2. Other experiments showed that up to three times as much chromogen could be formed in the presence of five atmospheres O_2 pressure as compared to air. The formation of chromogen by microsomes required both TPNH and ADP at all pressures of O_2 tested. After 1 min. at 70° the system was inactive with respect to chromogen formation, as well as the concurrent oxidation of TPNH, indicating that these processes were enzyme-catalyzed. In biological materials, formation of a chromogen giving a colored complex with TBA is widely accepted as an indication of lipid peroxidation (Bernheim, 1948). If lipid alterations were occurring in this microsomal system, examination of the microsomal lipids from control and experimental systems might be expected to show some differences. The incubations were performed at 5 atmospheres of O_2 to enhance possibility of finding enzyme-catalyzed lipid alterations, since at this O_2 tension the amount of chromogen detected was increased two- to three-fold (Table I.) After incubation, the lipids were extracted from the control and experimental incubation systems and equivalent quantities of lipid phosphorus were subjected to thin-layer chromatographic analysis (Fig. 1). Marked losses of phosphatidyl ethanolamine (PE) had occurred in the experimental system. Slow

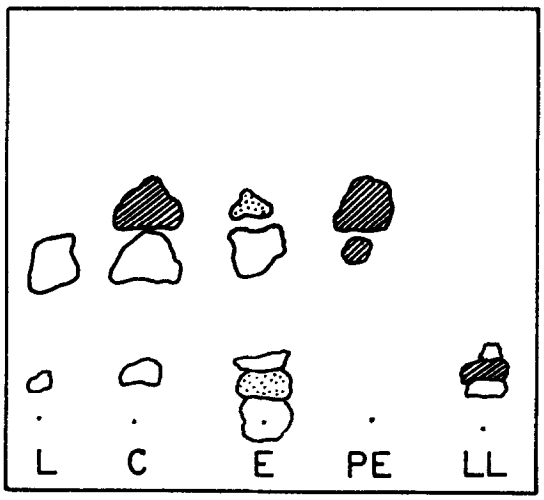

Figure 1. Thin-layer chromatography of total lipids from experimental and control systems. Solvent system: $CHCl_3$: MeOH:NH_4OH 75:25:4. (L) Lecithin, (C) Control lipid extract, (E) Experimental lipid extract, (PE) Phosphatidyl ethanolamine, (LL) mixture of lysophospholipids. Shaded spots were ninhydrin-positive, stippled spots were faintly ninhydrin-positive. Only spots positive for phosphorus are shown.

moving phospholipid compounds, which were present in the experimental lipid, but not in the control lipid appeared to be products due to the alteration of phospholipids. Gross analysis of these lipids are shown in Table II. No loss of extractable lipid phosphorus or ester groups

TABLE II

Analyses of Lipids from Control and Experimental Systems. Extraction of lipid: 20 vol. of $CHCl_3$:MeOH 2:1 were added to the incubation mixture (see Table I). Then 1/5 vol. of 0.5% NaCl solution was added and the chloroform layer was analyzed.

Lipid Analyzed	µg Lipid P / ml microsomes	Ester* P	Double Bonds* P		Amino N* P
Control	332	2.12	3.66[a]	3.44[b]	0.18
Experimental	315	2.02	2.58[a]	2.48[b]	0.12

* Ratios of total microequivalents in lipid extracts of incubation systems.
a Determined by bromination.
b Determined by catalytic hydrogenation.

was apparent. However, loss of double bonds, measured by both bromination and catalytic hydrogenation, as well as loss of amino nitrogen were apparent in the experimental lipid. Analyses of methyl esters from control and experimental lipids were performed by gas-liquid chromatography (Table III). Major decreases in polyunsaturated fatty acids (Table III) were observed in the experimental lipids, this being in agreement with the loss of double bonds as measured by bromination

TABLE III

Fatty Acid Composition of Total Lipids. Methyl esters were prepared by transmethylation and analyzed by gas-liquid chromatography (6 ft. column, butane diol succinate, 8%, 190-210°).

Lipid Analyzed	Wt. Per cent of Total Fatty Acids							
	16:0	18:0	18:1	18:2	20:0	20:4	22:0	22:6
Control	19.7	27.9	11.4	16.6	----	21.1	----	3.3
Experimental	25.4	34.5	13.6	16.4	----	9.3	----	0.9
Hydrogenated Control	23.0	48.1	----	----	24.1	----	4.4	---
Hydrogenated Experimental	36.6	54.9	----	----	8.9	----	Trace	---

(Table II). In this work, the control and experimental incubation systems were extracted with $CHCl_3$-MeOH according to the method of Folch et al. (1957). Addition of 0.5% NaCl resulted in the formation of an aqueous-methanol and a chloroform solvent layer. Aldehydes, determined by the dinitrophenylhydrazone method of Henick, (1954), were detected in both solvent layers derived from the extraction of the experimental system. In another test, the systems were incubated and then centrifuged to remove the microsomes. The supernatant of the experimental system was found to contain significant amounts of aldehydes and the TBA chromogen. Loss of double bonds from the total lipid and presence of aldehydes that are distributed between the aqueous-methanol and chloroform layers suggest chain scission of fatty acids, probably those associated with phospholipids. Loss of lipid amino-nitrogen without loss of lipid phosphorus (Table II) indicates that phosphatidyl ethanolamine is altered, but not to such an extent that it could not be extracted as a phospholipid. According to the currently accepted mechanism of peroxidation, chain scission of the fatty acid moiety of a phospholipid would result in formation of phospholipid-bound aldehydes. Compounds of this type could be expected to move slowly on thin-layer chromatographic plates. Since thin-layer chromatography demonstrated large losses of PE, it was of interest to determine if lipid alterations were specific for a particular phospholipid class. Lipids were extracted from large control and experimental incubation systems (300 ml) and chromatographed

on silicic acid, by elution with increasing concentrations of MeOH in $CHCl_3$. Fractions were pooled into the various phospholipid classes. Only 60% as much PE was present in the experimental lipid as compared to the control. Comparison of the fatty acid composition of each phospholipid class from the experimental lipid with that from the corresponding fraction in the control lipid showed that losses (50% or greater) of arachidonic and docosahexaenoic acids occurred in each phospholipid class from the experimental lipid, except the spingomyelin and lysolecithin fractions. The recovery from the columns was 96.5% and 87% for the control and experimental lipids respectively.

The results reported here indicate that under these experimental conditions, alterations of microsomal lipids occurred only during the enzymic oxidation of TPNH. These alterations seem to be selective toward arachidonic and docosahexaenoic acids present in phospholipids, but seem not to be selective for a given phospholipid class. The data presented are consistent with but do not prove the hypothesis that the lipid changes are due to peroxidation.

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