Reduced Triphosphopyridine Nucleotide Oxidase-catalyzed Alterations of Membrane Phospholipids

II. ENZYMIC PROPERTIES AND STOICHIOMETRY *

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SUMMARY

A study of the TPNH-dependent utilization of the polyunsaturated fatty acids in the phospholipids of the microsomal membranes has shown that the process is enzymic in nature. TPNH is oxidized during the reaction and O₂ is consumed. When all of the nucleotide is oxidized, both the lipid changes and the oxygen consumption stop. Addition of more TPNH (or a TPNH-regenerating system) reinitiates these activities. Parameters affecting the course of the reaction have been investigated, including studies with a number of inhibitors. The stoichiometry of the system has been determined with regard to TPNH, polyunsaturated fatty acid, and oxygen. Malondialdehyde is produced during this reaction, but it has been shown to be insignificant in terms of accounting for the polyunsaturated fatty acids which are utilized. The system bears some resemblance to that which synthesizes prostaglandin.

TPNH oxidase activity in normal rat liver microsomes has been shown to cause significant, reproducible changes in the composition of the endogenous phospholipids of microsomal membranes (1). The alterations involve the loss of major portions of portions of the polyunsaturated fatty acids situated at the β position of the phospholipids. Considerable oxygen is taken up during this reaction. Small amounts of malondialdehyde are formed, and the amount formed has been shown to be an index of the extent of the reaction (2). The system which promotes these lipid alterations is probably an integral part of the microsomal electron transport complex which catalyzes the oxidative metabolism of drugs and other organic substances foreign to mammalian cells (3). The studies described in the present report were designed (a) to establish the lipid alterations are the result of enzymic activity, (b) to investigate the conditions required for maximum activity, (c) to observe the effect of various inhibitors, and (d) to determine the stoichiometry of the system insofar as possible.

EXPERIMENTAL PROCEDURE

Materials

Animals—Adult male albino rats originally derived from the Holtzman-Sprague-Dawley strain were used. The animals were fed a commercial laboratory ration obtained from Rockland Laboratories, Teckland, Inc., Monmouth, Illinois.

Reagents and Solvents—All chemicals were reagent grade and were used as obtained except where specified. TPN⁺, TPN⁻, sodium D-glucose-6-P, yeast, glucose-6-P dehydrogenase (Type VI; yeast) and HMB were obtained from Sigma Chemical Company, St. Louis, Missouri. ADP was a product of P-L Biochemicals, Milwaukee, Wisconsin. Tris, chloroform, methanol, other organic solvents, and inorganic salts were purchased from Fisher Scientific. EDTA and 2-thiobarbituric acid were obtained from Eastman. Boron trifluoride-methanol reagent and some fatty acid methyl ester standards were products of Applied Sciences Laboratories, State College, Pennsylvania. Some methyl ester standards were purchased from the Hormel Institute, Austin, Minnesota. The “chromatoquality” reagent, n-hexane, was obtained from Matheson Coleman and Bell (Division of the Matheson Company, East Rutherford, New Jersey).

Methods

Microsomes were prepared as described in the accompanying paper (1).

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1 The abbreviations used are: HMB, p-hydroxymercuribenzoate; MDA, malondialdehyde; polyunsaturated-FA, polyunsaturated fatty acids.
**Enzyme Incubation Systems**—Assay conditions are given in each table or figure. All quantities of addition to the assay system are given as the final concentration values in the incubation system. Microsomes present in the reaction systems contained about 1 mg of protein per ml of final incubation volume, except in the case of the microsome concentration study (Fig. 5, below).

**Photometric Assays**—MDA formation was estimated by the thiobarbituric acid reaction (4) with 1-ml reaction systems incubated in a Dubnoff apparatus as described previously (1). TPNH oxidation was also determined with 1-ml reaction systems incubated in the same way. After removal of protein with 3 volumes of ethanol, the absorbance change at 340 nm was measured in a Beckman DU-2 spectrophotometer.

**Fatty Acid Analyses and Oxygen Uptake**—Systems (5 ml) were incubated in a differential respirometer for 15 min, or until thermal equilibrium was established. The reaction was initiated by tipping the substrate (TPNH or a mixture of TPNH, glucose-6-P, and glucose-6-P dehydrogenase) into the reaction chamber from the vessel side arm. Oxygen uptake was recorded until the indicated incubation time had elapsed. The vessels were promptly removed from the respirometer and the reaction was stopped by the addition of 25 ml of methanol. After the small quantity of silicone grease had been carefully removed from the ground glass fitting of the vessel, the contents of the flasks were transferred to 250-ml separatory funnels. The incubation vessels, which contained some residue, were then filled with chloroform-methanol (2:1, v/v). After the residual material had been extracted for 15 min, the solvent was transferred to the appropriate separatory funnel. The chloroform and methanol were then added to the separatory funnels, so that the final solvent composition was chloroform-methanol (2:1) and the ratio of the solvent volume to the original incubation volume was 20:1. This extraction procedure is essentially the same as that of Folch, Lees, and Sloane Stanley (5). At this point exactly 300 μg of highly purified erucic acid (22:1)* were added to each system as an internal standard. After the extraction systems were shaken thoroughly to ensure complete mixing of the internal standard and complete extraction of lipid from the microsomal membranes, 20 ml of 0.5% (w/v) aqueous NaCl solution were added to each separatory funnel. The mixture was shaken and, after phase separation, the lower (chloroform) layer was isolated and evaporated under reduced pressure. The lipid residue from each system was then taken up in 2 ml of chloroform. Aliquots were removed for various chemical analyses and for the preparation of fatty acid methyl esters for gas-liquid chromatography. The methyl esters were prepared by the boron trifluoride method (6).

**Fatty Acid Composition**—The fatty acid compositions of the total lipids extracted from the incubation systems were determined by gas-liquid chromatography of the methyl esters derived from the lipid. A description of the instrument conditions and the polyester columns used is given in Table II of the preceding paper (1). Methyl esters were identified by comparison of their relative retention times with those of known standard esters chromatographed under the same conditions. The identity of esters was confirmed by comparison of the retention times of hydrogenated sample esters with those of standard saturated esters as described in the preceding paper (1).

### RESULTS

**Enzymic Nature of MDA Production**—The chromatogram produced in this enzyme system yields a colored derivative when allowed to react with 2-thiobarbituric acid. The chromogram has been shown to be MDA (2). The enzymic nature of MDA production is shown in Figs. 1 to 3. Both MDA production by microsomal TPNH oxidase (Fig. 1A) and the oxidation of TPNH itself (Fig. 1B) were sensitive to mild heat treatment. Almost complete loss of these activities occurred when microsomes were heated for 30 sec at 65°C. It was also observed that continuous oxidation of TPNH was required for MDA formation (Fig. 2). Fig. 2, A and B, represents an experiment in which the formation of MDA (A) and the oxidation of TPNH (B) were followed simultaneously.

**Correlation among MDA Formation, O₂ Uptake, and Polyunsaturated FA Loss**—It was reported in the preceding paper (1) that microsomal TPNH oxidase catalyzes the conversion of the polyunsaturated fatty acids present in the microsomal membrane phospholipids to unknown moieties. A typical experiment which shows the time relationship among MDA formation, O₂

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*Number preceding colon indicates chain length; number following colon indicates the number of double bonds present.*

![Fig. 1. Heat sensitivity of microsomal TPNH oxidase and malondialdehyde (MA) formation.](image-url)

**Minutes**

|        | MDA Formation | O₂ Uptake |
|--------|---------------|-----------|
| 0      |               |           |
| 5      |               |           |
| 10     |               |           |
| 15     |               |           |

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Fig. 2. Dependence of malondialdehyde (MA) formation on continuous TPNH oxidation. A, malondialdehyde formation; B, corresponding TPNH levels. Incubation systems of experiments resulting in Curves 1A and 1B contained microsomes (approximately 1 mg of protein per ml of incubation system), 4 mM ADP, 0.012 mM FeCl₃, 10 mM nicotinamide, and 0.3 mM TPNH, all in 0.1 M Tris-HCl buffer, pH 7.5. For Curves 2A and 2B, the incubation system was the same as in 1A and 1B except that an additional quantity of TPNH (0.3 mM) was added at 10 min. For Curves 3A and 3B, the incubation system was the same as in Curves 1A and 1B except that 5 mM glucose-6-P and 0.5 Kornberg unit of glucose-6-P dehydrogenase per ml of incubation mixture were added at 10 min in order to regenerate TPNH.

uptake, and the amount of polyunsaturated-FA lost during TPNH oxidase activity is shown in Fig. 4A. On a molar basis, the final amount of O₂ uptake was about 4 times polyunsaturated-FA loss. Thus, O₂ uptake, and polyunsaturated-FA loss were qualitatively related but the quantitative relationship was complex and changed during the time course of the enzyme reaction.

Effect of Microsome Concentration on MDA Formation and O₂ uptake, and total polyunsaturated-FA loss (Fig. 4B), the three curves were similar. All of them were hyperbolic in shape. Thus, MDA production, O₂ uptake, and polyunsaturated-FA loss were qualitatively related but the quantitative relationship was complex and changed during the time course of the enzyme reaction.

Fig. 3. Effect of HMB on microsomal O₂ uptake and malondialdehyde (MA) formation associated with TPNH oxidase. A, no HMB added; B, 1 mM HMB added after 5 min of incubation; C, 1 mM HMB added at zero time. O₂ uptake (100% = 1068 mmole of O₂ uptake per mg of protein); MA, malondialdehyde formation (100% = 116 mmole of malondialdehyde per mg of protein). Data were normalized. Incubation system contained microsomes (approximately 1 mg of protein per ml of incubation mixture), 4 mM ADP, 0.012 mM FeCl₃, 10 mM nicotinamide, 0.3 mM TPNH, 5 mM glucose-6-P, and 0.5 Kornberg unit of glucose-6-P dehydrogenase per ml of incubation mixture, all in 0.1 M Tris-HCl buffer, pH 7.5. The incubation volumes were 1 ml for malondialdehyde assays and 5 ml for O₂ uptake.

Fig. 4. Time course correlation among O₂ uptake, polyunsaturated fatty acid (FA) losses, and malondialdehyde (MA) formation during the action of TPNH oxidase. A, time course, in millimicromoles per mg of protein; B, data normalized. O₂ uptake; MA, total polyunsaturated fatty acid lost; MA, malondialdehyde formation. Incubation system and conditions were the same as in Fig. 3. Polyunsaturated fatty acid losses and O₂ uptake were determined on the same 5-ml system; malondialdehyde was determined in 1-ml systems. The temperature was 37°C.

Fig. 5. Effect of the concentration of microsomes on malondialdehyde (MA) formation and O₂ uptake. The conditions and composition of the incubation systems were the same as in Fig. 3. Microsomal suspensions contained approximately 10 mg of protein per ml of suspension. A, 0.05 ml of microsomes per ml of incubation mixture; B, time course of malondialdehyde formation with three concentrations of microsomes; C, corresponding levels of TPNH.
The incubation system contained 0.5 ml of microsomes (approximately 5 mg of protein), 4 mM ADP, 0.012 mM FeCl₃, 10 mM nicotinamide, 6 mM glucose-6-P, and 2.5 Kornberg units of purified glucose-6-P dehydrogenase. All components were prepared in 0.1 M Tris-HCl buffer, pH 7.5, and the same buffer was used to bring the incubation systems to final volume. Incubations were carried out at 37º in air for 60 min in a 5-ml incubation volume. Lipids were isolated from each incubation system, and the fatty acid composition of each was determined as described under “Methods.” The amount of fatty acid was calculated from the gas chromatograms with the addition of an internal standard (methyl erucate) as described under “Methods.” Data are expressed as micromoles of each fatty acid present in the total lipid derived from the entire volume of each incubation system. Confidence in the values obtained was gained by calculating the ratio of total microsomal fatty acid methyl ester determined by this method to the microsomes of total lipid phosphorus in the incubation system. Essentially all of the fatty acids of rat liver microsomes are in the phospholipid fraction. For seven experiments the total fatty acid to lipid phosphorus value was 1.97 ± 0.05. In addition, for seven experiments, the ratio of total phospholipid fatty acid ester groups determined directly by the hydroxylamine method (8) to total lipid phosphorus was 2.06 ± 0.2. Thus, the value obtained by quantitative gas chromatography is in good agreement with the chemically determined value.

Fig. 6. Effect of TPNH concentration on the initial rates of MDA production. Incubation system and conditions were as in Fig. 3. The velocity (v) is expressed as millimicromoles of MDA formed per min per mg of protein. TPNH levels were varied from 0.6 to 300 µM. TPNH was maintained in the reduced state at all concentrations by means of the TPNH-generating system.

Table I

| TPNH added | 16:0 | 18:0 | 18:1 | 18:2 | 20:4 | 22:6 |
|------------|------|------|------|------|------|------|
| µM         |      |      |      |      |      |      |
| None       | 0.86 | 0.56 | 0.84 | 0.64 | 0.51 | 0.16 |
| 0.6        | 0.84 | 0.56 | 0.84 | 0.64 | 0.51 | 0.16 |
| 12         | 0.84 | 0.56 | 0.84 | 0.64 | 0.51 | 0.16 |
| 300        | 0.84 | 0.56 | 0.84 | 0.64 | 0.51 | 0.16 |

Fig. 7. The effect of incubation temperature on malondialdehyde (MA) formation. The incubation system was the same as in Fig. 3. The incubation temperatures were 0º; △, 21º; ○, 37º.

Experiments 1 and 2. These data represented the maximum amounts of polyunsaturated-FA loss under the conditions given (see Fig. 4), and implied nothing with regard to rates of polyunsaturated-FA loss. Effect of ADP-Fe³⁺+: Experiment 2 shows that there were only minor quantitative differences in polyunsaturated-FA lost when the lipid compositions of systems incubated in the absence and in the presence of ADP-Fe³⁺⁺ were compared. Thus, ADP-Fe³⁺⁺ was also not required for the
bation of microsomes without TPNH (control system) had no
effect for at least 45 min. Experiment 5 shows that a 60-min incu-
bation at 0.04 atm of O₂ pressure displayed a linear O₂ uptake
plateau (see Fig. 50) by about 30 min. However, systems in-
exhibited a hyperbolic oxygen uptake curve which was reaching a
maximum plateau though at a somewhat slower rate. Systems incubated in air
showed a similar uptake pattern, indicating that the oxygen uptake
was not affected by the air-nitrogen (1:4) mixture used to produce 0.04 atm of O₂
pressure. The incubation period was 60 min except in Experiment 3, in which it was 30 min. All other incubation parameters
are given in the table. Each experiment represents different
preparations of microsomes which show some variation in fatty
acid composition and protein content per volume. Lipids were
extracted and the fatty acids were analyzed as described under
"Methods."

| Incubation system | Temperature | Fatty acid composition* | O₂ uptake* |
|-------------------|-------------|------------------------|------------|
|                   |             | 16:0  | 18:0  | 18:1  | 18:2  | 20:4  | 22:6 |
| Basal (heated microsomes) | 37          | 1.85  | 1.90  | 0.87  | 1.50  | 1.57  | 0.51  |
| Basal plus TPNH-generating* | 37         | 1.75  | 1.88  | 0.88  | 1.20  | 0.62  | 0.16  |
| Basal, no incubation | 37          | 1.50  | 1.55  | 0.61  | 1.15  | 1.49  | 0.60  |
| Basal plus TPNH-generating* | 37         | 1.50  | 1.56  | 0.71  | 0.98  | 0.79  | 0.21  |
| Basal, no incubation | 37          | 1.50  | 1.56  | 0.71  | 0.98  | 0.79  | 0.21  |
| Basal | 37           | 1.51  | 1.74  | 0.56  | 0.95  | 1.38  | 0.48  |

* The fatty acid composition was determined as in Table I. Values represent the quantity of each fatty acid in the total 5-ml incubation volume.

Table II: Variation of incubation parameters and their effects on O₂ uptake and polyunsaturated-FA losses from microsomal lipids

Basal incubation systems consisted of 0.5 ml of microsomes (approximately 5 mg of protein), 4 mM ADP, 0.012 mM FeC₁₅₃, and
10 mM nicotinamide. All components were prepared in 0.1 M Tris-HCl buffer, pH 7.5. The same buffer was used to bring the incubation systems to final volume. Incubations were carried out in 5-ml volumes in air (except in Experiment 4, in which an air-nitrogen (1:4) mixture was used to produce 0.04 atm of O₂). ADP-Fe⁺⁺ was routinely added, however, since it had an effect
on rates and for the sake of uniformity of experimental conditions.

Heat inactivation: The effect of heating microsomes at 65°C for 2 min is shown in Experiment 3. When the lipid composition of control systems containing heated microsomes was compared with that of systems containing untreated microsomes, it was apparent that the heat treatment had little if any effect
on the polyunsaturated-FA content. Furthermore, the lipid from heated microsomes incubated in the presence of TPNH and a TPNH-generating system showed only losses of polyunsaturated-FA when compared to its control and the fully active system. Thus, the activity responsible for the polyunsaturated-FA losses, as well as for MDA formation and O₂ uptake, is
heat sensitive. Reduced O₂ pressure: Experiment 4 shows that the losses of polyunsaturated-FA also occurred at 0.04 atm, al-
though at a somewhat slower rate. Systems incubated in air exhibited a hyperbolic oxygen uptake curve which was reaching a plateau (see Fig. 5D) by about 30 min. However, systems incubated at 0.04 atm of O₂ pressure displayed a linear O₂ uptake for at least 45 min. Experiment 5 shows that a 60-min incu-
bation of microsomes without TPNH (control system) had no effect on the fatty acid composition, since the latter was essen-
tially identical with that of nonincubated microsomes. Thus, the
TPNH-dependent reaction utilizing polyunsaturated-FA from microsomal membrane lipid takes place under very mild tem-
perature conditions, at low O₂ pressures, and at very low TPNH concentrations (see Table I). All conditions and requirements
for the reaction were at least compatible with physiological parameters whether the reaction takes place in vivo or not, unless it is argued that microsomes in vivo are not in a physi-
ological state.

Effects of Inhibitors on MDA Formation—HMB is an inhibitor of microsomal electron transport (9-11) and also of lipid peroxi-
dation (12). The effects of various kinds of inhibitors on MDA formation are shown in Table III. Since inhibition by HMB is
considered to be the result of a reaction with essential sulfhydryl groups, other sulfhydryl reagents were also tested. HMB not
only prevented formation of MDA when added at zero time, but it also immediately stopped formation of MDA when added
after the initiation of the MDA-forming reaction. However, other sulfhydryl reagents, such as N-ethylmaleimide, iodoacetate,
and iodoacetamide, had little or no effect on MDA formation. Thus, the effect of HMB may or may not be an effect on sulfhy-
dryl groups. Inorganic cations, such as Mn⁺⁺ and Co⁺⁺, are known to inhibit lipid peroxidation reactions (13). Although

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TABLE III

Inhibitors of MDA formation in microsomal TPNH oxidase system

The control incubation system contained 0.1 ml of microsomes (approximately 1 mg of protein), 4 mM ADP, 0.012 mM FeCl₃, and 10 mM nicotinamide, all in 0.1 M Tris-HCl, pH 7.5. The experimental system contained all components of the control plus 0.3 mM TPNH, 6 mM glucose-6-P, and 0.5 Kornberg unit of glucose-6-P dehydrogenase. Incubations were carried out in air at 37°C in 1-ml incubation volumes. All inhibitors were present at a level of 1 mM except α-tocopherol and sodium sulphite (50 mM).

| Inhibitor | Time of inhibitor addition (min) | Total incubation time (min) | MDA formed | Experimental system | Control system |
|-----------|---------------------------------|-----------------------------|------------|---------------------|----------------|
| None      | 0                               | 0                           | 5          | 5                   | 5              |
| None      | 5                               | 29                          | 6          | 6                   | 6              |
| None      | 15                              | 68                          | 5          | 5                   | 5              |
| HMB       | 0                               | 15                          | 5          | 14                  | 13             |
| N-Ethylmaleimide | 0       | 15                          | 5          | 29                  | 30             |
| Iodoacetamide | 0     | 15                          | 5          | 29                  | 36             |
| Iodoacetate | 0     | 15                          | 5          | 29                  | 61             |
| Mn²⁺      | 0                               | 15                          | 5          | 52                  | 54             |
| Co³⁺      | 0                               | 15                          | 5          | 50                  | 52             |
| EDTA      | 0                               | 15                          | 5          | 46                  | 46             |
| None      | 0                               | 15                          | 5          | 29                  | 29             |
| Sulfité   | 0                               | 30                          | 0          | 7                   | 7              |
| None      | 0                               | 20                          | 0          | 2                   | 2              |
| α-Tocopherol | 0     | 20                          | 0          | 2                   | 2              |

* α-Tocopherol was introduced by homogenization with the liver at the time of preparation of microsomes (12.5 mg of α-tocopherol per g of liver).

Both of these cations prevented MDA formation, there did not appear to be an instantaneous inhibition of this process by these cations when the latter were added 5 min after the initiation of the MDA-forming reaction. Similarly, EDTA was able to prevent MDA formation if added prior to the addition of TPNH, but did not stop MDA production immediately if added 5 min after TPNH was added. α-Tocopherol was also able to prevent MDA formation by the TPNH oxidase system when it was homogenized with the liver during preparation of the microsomes (13). The effect of dietary α-tocopherol on this enzyme system is the subject of a forthcoming report.  

Sulfite is known to be oxidized aerobically by a free radical mechanism (12), and has been used to detect free radical intermediates produced by certain enzyme reactions (14). Addition of small quantities of sulfite, which were insufficient to maintain free radical reactions necessary for its oxidation, were without effect on MDA production. However, higher concentrations of sulfite (12 mM) were able to reduce MDA formation greatly and were also able to sustain the free radical reactions required for sulfite oxidation. These data implied that sulfhydryl groups, a metal, and a free radical are probably involved in some part of the enzyme system.

Effect of Inhibitors on Polyunsaturated-FA Losses—Table IV shows the effect of Co²⁺ and HMB on the amount of polyunsaturated-FA lost from microsomal membrane lipids as a result of the oxidation of TPNH. Experiment 1 shows that Co²⁺ caused very little inhibition of the polyunsaturated-FA-utilizing activity at low concentration (0.01 mM). However, polyunsaturated-FA utilization was markedly inhibited when the system contained 1 mM Co²⁺. The residual O₂ uptake observed with the higher level of Co²⁺ was presumably due to the oxidation of TPNH, which is independent of lipid utilization. The effect of the addition of HMB to the enzyme system is shown in Experiment 2. The O₂ uptake was completely stopped and the polyunsaturated-FA losses were greatly reduced by HMB. Other experiments have shown that HMB induces some MDA formation in the control incubation system (see Table III), and thus small polyunsaturated-FA losses may occur in the presence of HMB. These experiments confirm the close relationship among polyunsaturated-FA losses, O₂ uptake, and MDA formation, and are in agreement with the enzymic nature of the reactions.

Time Course of Polyunsaturated-FA Losses Fig. 8 shows the time course of the fatty acid composition of lipids from microsomes actively oxidizing TPNH. Duplicate values are shown, and it can be seen that reproducibility was reasonably good. The content of 16:0, 18:0, and 18:1 in the system remained constant throughout the time course of the reaction. The value of 18:2 decreased somewhat during the 15-min period depicted, but greater losses are known to occur at 30 and 60 min. Both 20:4 and 22:6 were 50% utilized at the end of the 15-min incubation period. Since the molar quantity of each fatty acid in the system could be determined, it was clear that the contents of saturated and monounsaturated fatty acids were not altered by this reaction. The process appeared to be specific for the more highly unsaturated fatty acids, especially 20:4 and 22:6.

Stoichiometric Studies—The stoichiometric relationships among polyunsaturated-FA loss, TPNH oxidation, and O₂ uptake are shown in Table V. All quantities are expressed on the basis of microsomal protein. The loss of each fatty acid for the 60-min time period is shown for five separate experiments. The total polyunsaturated-FA lost was taken as the sum (in millimicro moles) of the losses of 18:2, 20:4, and 22:6. Losses of other polyunsaturated-FA, present in the microsome in trace amounts, were quantitatively insignificant. Comparison of the molar ratios tabulated in the last column of Table V divulges that the stoichiometry was rather consistently fatty acids to TPNH to O₂, 1:1:4. The 1:1 ratio between O₂ uptake and polyunsaturated-FA loss shown in Table V, however, was a limiting value, and the time relationship between these two quantities is shown in Fig. 9. The O₂ to fatty acid ratio changed throughout the time course of the reaction, and approached the limiting value of approximately 4. As indicated in Fig. 9, when the ratio was extrapolated to zero time, the value was approximately 1.

Table VI shows that there was good agreement between the number of double bonds lost calculated from polyunsaturated-FA disappearance and the actual number lost, as indicated by bromination (15). This indicated that the product contained none of the unsaturation of the polyunsaturated-FA precursors, and was in general agreement with previous hydrogenation studies (16).
TABLE IV

Effect of inhibitors on polyunsaturated-FA losses and O$_2$ uptake

Each incubation system contained 0.5 ml of microsomes (approximately 5 mg of protein), 4 mM ADP, 0.012 mM FeCl$_3$, and 10 mM nicotinamide in 0.1 M Tris-HCl, pH 7.5. All other additions are given in the table. Inhibitors were added at zero time. Incubations were carried out in air at 37° in 5-ml incubation volumes.

| Fatty acid composition | O$_2$ uptake $^a$ |
|------------------------|------------------|
| 16:0 18:0 18:1 18:2 20:4 22:6 | $\mu$moles |
| 1.58 1.84 0.77 1.15 1.21 0.31 | 0.3 |
| 1.39 1.52 0.67 0.65 0.19 0.09 | 0.8 |
| 1.43 1.53 0.69 0.47 0.22 0.14 | 0.4 |
| 1.37 1.66 0.69 1.02 1.13 0.16 | 2.1 |

$^a$ TPNH (0.3 mM), 6 mM glucose-6-P and 2.5 Kornberg units of glucose-6-P dehydrogenase were added where indicated.

TABLE V

Stoichiometry of TPNH oxidase-catalyzed utilization of polyunsaturated-FA

Each incubation system contained 0.5 ml of microsome (approximately 5 mg of protein), 4 mM ADP, 0.012 mM FeCl$_3$, 10 mM nicotinamide, and TPNH (0.33 mM in Experiments 1 to 4; 1.0 mM in Experiment 5), in 0.1 M Tris-HCl, pH 7.5. Incubations were carried out in air for 60 min at 37° in 5 ml volume. Lipid isolation and analysis were carried out as described under “Methods.” Fatty acid utilization was determined by the difference between the fatty acid content of the incubated control (no TPNH) and the system that contained TPNH. The total fatty acid utilized was taken as the sum of 18:2, 20:4, and 22:6 consumed. TPNH oxidation and O$_2$ uptake were measured as described in “Methods.”

| Fatty acid utilized | TPNH oxidized | O$_2$ uptake | Molar ratio, fatty acid to TPNH to O$_2$ |
|---------------------|---------------|--------------|---------------------------------------|
| 16:0 20:4 22:6 Total | $\mu$moles/mg protein |
| 18:2 | 20:4 | 22:6 | Total |
| 1 | 6 | 234 | 72 | 312 | 330 | 1230 | 1:1.06:3.94 |
| 2 | 57 | 184 | 53 | 294 | 330 | 1220 | 1:1.12:4.15 |
| 3 | 50 | 188 | 57 | 295 | 330 | 1210 | 1:1.12:4.10 |
| 4 | 118 | 174 | 42 | 334 | 330 | 1240 | 1:0.99:3.71 |
| 5 | 129 | 222 | 45 | 396 | 356 | 1445 | 1:0.87:3.65 |

Fig. 8. Relation between TPNH oxidase incubation time and the fatty acid (FA) composition of microsomal lipids. Incubation system and conditions were the same as in Fig. 3. The incubation volume was 5 ml for each point. After the indicated incubation time, lipid was extracted, isolated, and analyzed as described under “Methods.” Incubations were run in duplicate to show the reproducibility of the method. Data are expressed as millimicromoles of fatty acid present in the total lipid extract per mg of protein in the incubation system. Unincubated control values are the zero time values.

Since it was known that carbonyl compounds were among the products formed in the TPNH oxidase-catalyzed lipid alteration reactions (1), it was thought that O$_2$ might be utilized to further oxidize aldehydes to acids. With this possibility in mind, an experiment was done in which semicarbazide was added to the reaction system as a carbonyl “trap” to prevent a possible further oxidation. Accordingly, that portion of the O$_2$ consumed which might be associated with the postulated oxidation should be prevented. Table VII shows that semicarbazide had no effect on the O$_2$ to fatty acid stoichiometry. It may be argued that semicarbazide, owing to solubility or steric factors, might be excluded from reacting with the carbonyl compounds. However, as already shown in these studies, other organic substances and inorganic cations apparently penetrated the microsome sufficiently to act as inhibitors of the system. Semicarbazide has been used in other enzyme systems to trap carbonyl compounds (17).

DISCUSSION

Three aspects of the phenomenon described above and in the preceding paper (1) seem particularly significant. First, essentially all of the fatty acids involved in the reaction are situated at the $\beta$ position of microsomal phospholipids. Second, the conditions under which the membrane lipids are rapidly altered may
exist in a number of TPNH-requiring enzymic assays in which the microsomal fraction of rat liver cell particulates is utilized as an enzyme. The influence, therefore, of possible extensive and rapid changes in the microsomal lipid composition on such systems is unknown. It would seem particularly pertinent to determine possible effects in enzymic systems containing microsomes and TPNH in which studies on fatty acid biosynthesis and interconversions are being carried out. Third, the conditions which resulted in maximum activity of the lipid-altering system could apparently exist in vivo. There is a possibility, therefore, that a role for this phenomenon in the turnover of the phospholipid portion of microsomal membranes may exist. Omura, Siekevitz, and Palade (18) have shown that the turnover of phospholipids in the microsomal membranes is somewhat more rapid than that of the protein portion.

Our studies have shown that the microsomal TPNH oxidase-catalyzed lipid changes were inhibited in vitro when the animals had been given very high dietary levels of α-tocopherol, but elimination of these high levels of α-tocopherol from the diet for as little as 7 hours totally reversed the inhibition observed in vitro (16). Studies from this laboratory (19) have also shown that 20:4 has a faster turnover rate in the liver of α-tocopherol-deficient animals relative to other fatty acids as compared to control animals. TPNH oxidase-catalyzed alterations of membrane lipids could account for this observation.

In spite of the quantity of the data shown in this report and in the preceding paper (1), the mechanism of the enzymic reaction utilizing polyunsaturated fatty acids of phospholipids remains obscure. It may be that all glycerophosphatides containing polyunsaturated FA are substrates for this reaction, but the data in these studies provide sufficient evidence only to show that at least phosphatidylethanolamine and phosphatidylcholine are utilized. The results indicate that the utilization of polyunsaturated-FA in the microsomal lipid is enzymic and occurs at a rapid rate. From 15 to 20% of the total fatty acid content of the phospholipid was commonly consumed in the reaction, most of which occurred during the first 15 min of the incubation. The lipid-altering reaction appears to be limited by the amount of polyunsaturated-FA present in the phospholipid, since 80 to 90% of these acids were utilized when the reaction was allowed to go to completion. The process was accompanied by O₂ consumption and the formation of a stoichiometrically small amount of MDA. That MDA is probably a minor (although consistent) product of this reaction can be supported by the fact that although over 400 nmol of fatty acid were utilized, only about 45 nmol of MDA were produced (see Fig. 4).

The production of MDA followed typical enzyme kinetic patterns. The double reciprocal Lineweaver-Burk plot was linear and gave an apparent $K_m$ of $1 \times 10^{-4}$ m. This value is of the order one would expect for a pyridine nucleotide, and is in essential agreement with values obtained by others for various microsomal TPNH-dehydrogenating enzyme activities (20). It may be noteworthy that essentially the same quantity of fatty acid was altered when microsomes were incubated in the presence of quantities of TPNH approximating the $K_m$ value (e.g. 0.6 μM, Table I) as at higher levels of TPNH (e.g. 300 μM) provided that a TPNH-regenerating system was added to maintain the nucleotide in the reduced state.

The studies with sulfhydryl reagents, EDTA, metal ions, and free radical-trapping agents imply (a) that the enzyme system contains a site (or sites) susceptible to HMB which may or may not involve a sulfhydryl group (or groups), (b) that a metal component is required, and (c) that a free radical component may be essential.

The time course study of polyunsaturated-FA utilization in this system indicates that loss of double bonds from microsomal

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**Table VI**

| Fatty acid lost | Double bonds | O₂ uptake | Ratio, O₂ to double bonds lost |
|----------------|-------------|-----------|-----------------------------|
|                | Calculated  | Measured  | Calculated                  | Measured                   |
| 18:2           | 0.36        | 0.72      |                            |                            |
| 20:4           | 1.24        | 4.96      |                            |                            |
| 22:6           | 0.48        | 2.88      |                            |                            |
| Total          | 2.08        | 8.56      | 8.00                        | 5.85                        | 0.68 | 0.73 |

**Table VII**

| Semicarbazide | O₂ to fatty acid ratio |
|---------------|------------------------|
| None          | 4.57                   |
| 10 mm         | 4.17                   |

---

**Fig. 9.** Time course of stoichiometric relation between O₂ uptake and polyunsaturated fatty acid (PUFA) losses. Incubation system and conditions were the same as in Fig. 3. Each point represents a 5-ml incubation system in which O₂ uptake and fatty acid analyses were done as described under “Methods.” Polyunsaturated-FA losses were the sum of the losses of 18:2, 20:4, and 22:6. The ratio given in this graph was determined by dividing the number of micromoles of O₂ uptake for a given system by the total micromoles of polyunsaturated FA lost in that system.
lipid cannot be explained on the basis of saturation reactions, since the amounts of the saturated acids remained constant. Staudinger and Zubrzycki (21) have reported that microsomes reduce unsaturated fatty acids under similar conditions.

The stoichiometric studies indicate that there is an initial 1:1 molar relationship between O₂ consumption and fatty acid loss from membrane lipids. This ratio continues to increase to a limiting value of approximately 4:1 as the reaction proceeds. The results indicate that an initial attack on lipid, requiring oxygen, occurs and that subsequent secondary reactions which consume O₂ must follow almost immediately, thereby modifying the initial product further. In order to gain some insight as to what a limiting stoichiometry of 4:1, O₂ to fatty acid, might mean, calculations of the total number of double bonds present in the fatty acids utilized were made according to Table VI. For over 20 experiments in which the incubation period was long enough so that O₂ uptake had ceased (i.e. the limiting condition), the ratio of O₂ to the lipid double bonds contained in the polyunsaturated-FA that were utilized was always 1:1. The agreement between these calculated double bond values and unsaturation values determined by bromination indicates that the products of the reaction contained none of the unsaturation of their polyunsaturated-FA precursors. It appears, therefore, that there is a stoichiometric relationship between O₂ uptake and actual double bonds lost or between O₂ uptake and some molecular feature of polyunsaturated-FA that is correlated with the number of double bonds in the molecule. At present it is not possible to say what portion of the O₂ taken up is actually incorporated into the lipid.

MDA is a minor product (on a molar basis, equivalent to approximately 12% of the fatty acid loss and only 3% of the O₂ uptake). It is also a minor product in lipid peroxidation in model systems (22). It may be pertinent to mention a similarity between this system and the biosynthesis of prostaglandins from polyunsaturated-FA. The latter system requires a donor of reducing equivalents, consumes O₂ utilizes polyunsaturated-FA, and produces stoichiometrically small quantities of MDA (28). It is fortunate that MDA formation and lipid alterations described in this work are closely correlated in this microsomal system, since the determination of MDA is simple and rapid, but we feel it is hazardous to design experiments and draw conclusions on the basis of an assay which measures as little as 3% of the products involved in a reaction.

Lipid peroxidation in model systems is auto-catalytic (24), whereas TPNH oxidase-catalyzed lipid alterations appear not to be. That the reaction is not auto-catalytic after the first oxygen attack is indicated in Fig. 9, which shows that once the enzyme reaction is stopped at any point during the process, the particular ratio of O₂ to fatty acid reached at the instant remains stable. All investigated methods of stopping enzymic oxidation of TPNH in this system also simultaneously stop the lipid alterations. The tight "coupling" between active TPNH oxidation and lipid alteration is probably related to the highly ordered structural nature of the microsomal membrane. For example, only a small amount of lipid from heated microsomes (60° for 30 sec) was altered when the heated microsomes were added to a system in which active microsomes were oxidizing TPNH.

There is evidence indicating that the system which promotes these lipid alterations is correlated with the activity of the microsomal electron transport system and, therefore, may have components in common with the TPNH requiring system which oxidizes certain types of drugs (3). The participation of some type of free radical produced by a component of this drug-metabolizing system seems certain (25). Furthermore, the addition of certain compounds which can be hydroxylated or demethylated by the drug-metabolizing system to microsomes being incubated with TPNH greatly reduces the amount of MDA produced (3). By extension of the ideas of Nilsson, Orrenius, and Ernster (25), we interpret this to mean that in the presence of a supply of TPNH, this microsomal electron transport system produces free radicals which react readily with certain types of metabolites and foreign organic compounds. In the absence of such substances, the radicals attack microsomal phospholipids at a rapid rate, specifically the fatty acids containing two or more double bonds, and particularly arachidonic acid. Monoenoic and saturated fatty acids appear unaffected.

Although one can do little more than speculate about a possible physiological role or pathological consequence of this lipid-altering system in the living animal at the moment, the TPNH requirements are in the physiological range. The formation of MDA (Fig. 7), losses of polyunsaturated-FA, and uptake of O₂ readily occur at reduced O₂ tensions (0.04 atm) and under very mild temperature conditions. An important point in that the addition of iron in the form of an ADP complex is not required.

Since abnormally high levels of dietary a-tocopherol completely inhibit the lipid-altering reaction in vitro (16), a biochemical explanation for at least part of the continuous requirement for this vitamin in animals may be forthcoming.

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