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Rolf J. Mehlhorn and Lester Packer

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DISCLAIMER

Classification: Biophysics

NITROXIDE DESTRUCTION AND FLAVIN-PHOTOSENSITIZED

DAMAGE IN INNER MITOCHONDRIAL MEMBRANES*

(Free Radicals/Lipid Peroxidation/Respiration)

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ABSTRACT

Nitroxide free radicals lost their paramagnetic absorption spectrum when they were illuminated with visible light in aged but not freshly isolated inner mitochondrial membranes (SMP's). The action spectrum of spin loss rates coincided with a flavin absorption spectrum. Spin loss was comprised of reduction and "destruction"; the latter being defined as spin loss that cannot be reversed by ferricyanide oxidation. By placing SMP's in gas permeable tubing and illuminating alternately under nitrogen and air, it was possible to discriminate qualitatively between spin reduction and destruction in the same sample. Aerobic spin loss was comprised entirely of destruction. When aged SMP's were centrifuged, spin loss was observed in supernatants but not pellets; hence aqueous factors appeared to cause spin loss. Flavin fluorescence was observed in the supernatants, suggesting that free flavins catalyzed spin loss. However, addition of exogenous flavins to fresh SMP's did not cause spin loss; hence some other factor together with flavins was required to cause nitroxide destruction. This factor accumulates during either aerobic or anaerobic aging of SMP's and may be present in mitochondria. Supernatants of aged SMP's, when added to freshly isolated SMP's, induced rapid nitroxide destruction slightly accelerated photodamage to succinate oxidase and considerably increased photo-induced lipid peroxidation, suggesting that photodamage by the bound flavin of succinate dehydrogenase occurs in close proximity to the binding site, whereas free flavins catalyze destruction of random membrane targets.

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ABBREVIATIONS

SMP	Submitochondrial preparations
EDTA	Ethylene diamine tetracetic acid
TBA	Thiobarbituric acid
TEMPOL	2,2,6,6-Tetramethyl-4-piperidinol-1-oxyl (see Materials and Methods for other nitroxide structures)
DMPO	5,5-Dimethyl-1-pyrroline-N-oxide
POBN	2-(4-Pyridyl 1-oxide)-N-tert-butylnitrone
PRETOL	2,2,6,6-Tetramethyl-4-piperidinol
SOD	Superoxide dismutase

INTRODUCTION

The destructive effect of visible light on tissues, cells and organelles has been studied extensively (1). Blue light is particularly damaging in many cases, suggesting flavin involvement. Previously, this laboratory reported on visible light effects in mitochondria (2,3). Progressive functional inactivation was observed, beginning with the loss of ATP synthesis, and followed by inactivation of dehydrogenase flavoenzymes. Functional damage was accompanied by loss of flavins , sulfhydryl groups and lipid peroxidation. Although light damage was enhanced by exogenous flavins, covalently bound flavins were destroyed at rates comparable to those for dissociable flavins. Furthermore, the inactivation required oxygen but it was unclear whether this requirement was merely to maintain the flavins in their light absorbing oxidized form or whether active oxygen species were directly responsible for damage. Failure to achieve protection with SOD, singlet oxygen scavengers, hydroxyl radical quenchers and antioxidants, suggested a minor role if any for these potentially destructive species except perhaps at the active sites of flavoenzymes (2).

Two aspects of the damage process interested us particularly because of the possibility of further studies using nitroxide spin probes. These were the possibility that damage was mediated by free radicals that might have been generated by the excited flavins and the role of bound vs. free flavins. Nitroxides are potentially useful to study oxygen involvement in free radical reactions because they are themselves free radicals, $N\text{-}O\cdot$, which resemble ground state oxygen, $\cdot O\text{-}O\cdot$, and which may thus compete with oxygen in free radical addition reactions. However, O_2 adducts are reactive peroxy radicals which may participate in chain reactions, whereas nitroxide spin pairing products are expected to be relatively unreactive. A number of stable nitroxide adducts

have been described (4,5). Nitroxides also have the potential to serve as free radical detectors in another sense, since they should readily undergo one-electron or hydrogen transfer reactions with other free radical molecules. One-electron transfer reactions of nitroxides have been demonstrated with reduced flavins (6), and with electron transport components of mitochondria (7). However, since reduction of nitroxides can occur by two electron reductants like ascorbate, loss of nitroxide paramagnetism as assayed by electron spin resonance is not sufficient to establish free radical involvement in an interaction per se, and additional analyses are required to establish such involvement.

In other detection schemes, nitroxide precursors and derivatives have been used to detect active oxygen species, including superoxide radicals (8,9) and singlet oxygen (10). These detection schemes exploit oxidation of the nitroxide derivatives to elicit a paramagnetic signal.

Mechanisms and biological effects of photosensitized oxidation were recently reviewed (11). The photooxidation of amino acids by flavins in particular has been studied extensively (e.g., 12,13). Analysis of the photoinactivation of a flavoenzyme, porcine D-amino acid oxidase provided evidence of localized destruction of amino acid residues at the flavin binding site (14). The light mediated reduction of many flavoproteins appears to be catalyzed by traces of free flavins, i.e., aqueous photoreductants generally do not appear to have direct access to enzyme-bound flavins (15).

In this report, the effect of illumination on nitroxides in mitochondrial membrane suspensions is described and correlated with photodamage to succinate oxidase of the respiratory chain and with light induced lipid peroxidation.

MATERIALS AND METHODS

Preparations: Rat liver mitochondria were isolated and suspended in 200 mM mannitol, 70 mM sucrose and 1 mM Tris, pH 7.4. They were washed twice in the same medium and finally resuspended in 30 mM potassium phosphate, pH 7.4. Submitochondrial preparations (SMP's) were prepared by intermittent sonication of the mitochondria with a Branson 350 cell disruptor at a power setting of 5 for a cumulative time of 2 minutes. Undisrupted mitochondria were removed by centrifugation at 8,500 x g for 10 minutes and the SMP's collected by centrifugation at 100,000 x g for 30 minutes. They were resuspended in 30 mM potassium phosphate, pH 7.4 at protein concentrations indicated in the figures.

Aging Procedure: SMP's were placed in 25 ml Erlenmeyer flasks, and the flasks were sheathed with aluminum foil and closed with a rubber stopper to prevent evaporation. In one experiment, flasks were flushed for 30 minutes with argon on an ice bath before being stoppered. The flasks were then placed on a shaking water bath at 37° C for the time periods indicated in the figures.

Nitroxides, some reduced derivatives and a precursor are shown below:

[Probe structures, p. 5a]

The designation, TEMPR, refers to the structure 4-R-2,2,6,6-tetramethyl piperidine-1-oxyl (except for TEMPO).
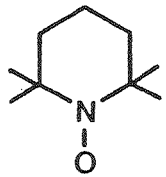
TEMPOL, TEMPONE, TEMPAMINE, PRETOL, DMPO and POBN were purchased from Aldrich Chemical Co. TEMPCARBOXYLATE (16) and CAT_1 (17) were synthesized.

TEMPO, TEMPOL and ^{15}N TEMPONE were generous gifts from A.D. Keith.

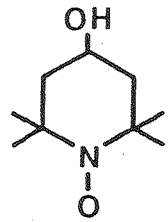[15]N TEMPOL was prepared by reducing ^{15}N TEMPONE with sodium borohydride and extracting the product into diethyl ether.

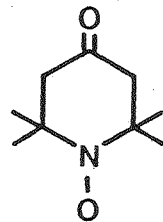The reduced nitroxides, TOLH, TAH and TCH were prepared by treating TEMPOL, TEMPAMINE and TEMPCARBOXYLATE, respectively, with ten equivalents of sodium
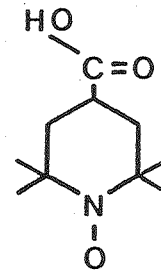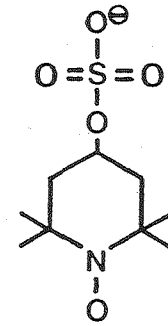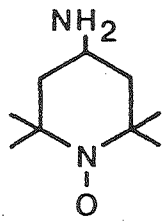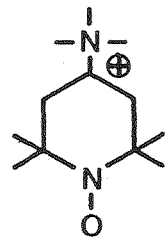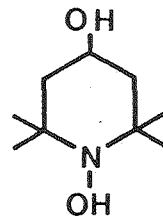
PROBES

TEMPO

TEMPOL

TEMPONE

TEMPCARBOXYLATE

TEMPSULFATE
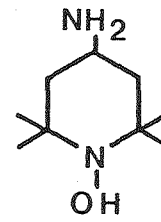
TEMPAMINE

CAT$_1$
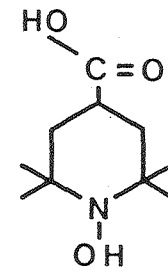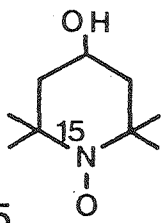
TOLH

TAH

TCH

^{15}N TEMPOL
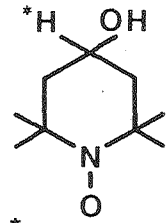
*TEMPOL
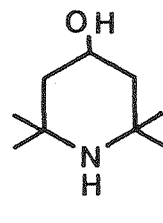
PRETOL
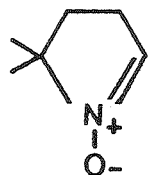
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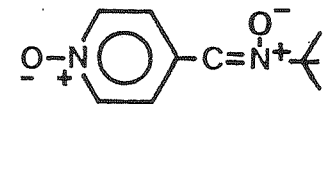
POBN

ascorbate at pH 7, adjusting the pH to 7, 11 and 3, respectively, and extracting the products into diethyl ether. The ether extracts were washed once with phosphate buffer solutions at pH 7, 11 and 3 as above, dried over Na_2SO_4 and the solvent was removed under reduced pressure.

TEMPSULFATE was prepared as follows: 3 g of TEMPOL and 4.45 g of N,N-diisopropyl-ethylamine were taken up in 100 ml of acetone. The solution was stirred rapidly on an ice bath and 1.76 g of chlorosulfonic acid were added over a period of 10 minutes. The reaction mixture was removed from the ice bath, allowed to stand at room temperature for 2 hours, treated with 1.4 g of NaOH in 20 ml of water and then the amine and unreacted TEMPOL were extracted exhaustively with diethyl ether. The water fraction was dried under an air stream and used without further purification.

ESR Experiments: Stock solutions of the probes in water were adjusted to pH 7.0 with HCl or NaOH. In a 0.6 ml test tube, 35 microliters of SMP's were mixed with 0.35 microliter of probe. Mixing was effected with hand agitation. Except for the action spectrum experiment, labeled samples were transferred to gas-permeable tubing (18) with a 100 microliter syringe, and then the flexible tubing was bent in the middle and inserted in a thin-walled quartz tube open at both ends. The pressure of the tubing on the walls of the quartz housing tube was sufficient to immobilize the sample within the ESR cavity even in the presence of a nitrogen stream. The action spectrum was obtained with samples in 50 μl glass capillaries sealed at one end with a propane torch. ESR experiments were conducted in a Varian E109E spectrometer at a power setting of 10 mW and a modulation amplitude of 1.25 gauss at a frequency of 100 KHz. A typical gain setting for a kinetic scan was 3.2×10^4.

Illumination: Samples were illuminated within the microwave cavity of the ESR instrument with a 100 W projector and a glass heat filter. The light

intensity at the sample was 150 mW/cm^2. The intensity was determined outside

the ESR instrument with a Hewlett-Packard 8330A radiant flux meter and a 8334A

detector and subsequently determined within the instrument by comparing FMN-

mediated oxidation rates of TOLH inside and outside the cavity under nonsatur-

ating conditions. The action spectrum of spin loss rates was determined using

Oriel interference filters of 10 nm band width (G-522-11) and normalizing spin

loss rates with respect to light intensities transmitted by the filters as

measured with the radiant flux meter. Except where white light is indicated

in the figure legends, all sample illumination in the ESR instrument was conducted

with an Oriel broadband interference filter with a peak position at 450 nm

and a 50 nm bandwidth. With this filter, FMN-mediated TOLH oxidation occurred

at 1/3 the rate observed with white light. For the ferricyanide-reoxidation

experiment, SMP's in an Erlenmeyer flask at room temperature were illuminated

with the same light source but the light was passed through a Corning glass

#3389 400 nm cutoff filter. The photodamage experiment was conducted at 10° C

in an apparatus that was described previously (3), providing an intensity of

300 mW/cm^2 with the Corning #3389 filter.

RESULTS

Description of the Spin Loss Phenomenon: A spectrum of TEMPOL in a suspension of SMP's is shown in the inset of Figure 1. The effect of visible light on this spectrum is to cause a decrease of the intensity of the spectrum, but only if the membranes have been allowed to incubate prior to illumination. As shown in Fig. 1, the onset of spin loss with illumination as well as its cessation in the dark are virtually instantaneous.

Action Spectrum: Spin loss rates at different wavelengths of light are shown in Figure 2. Because of the narrow bandwidths of the interference filters used for these experiments, effective light intensities were low and about 15 minutes of illumination were required to obtain measurable spin loss rates at some wavelengths. An absorption spectrum of flavin mononucleotide (FMN) is shown for comparison.

Direct Interactions Between Flavins and Nitroxides: The action spectrum of spin loss rates implicated flavins in the spin loss process observed in aged SMP's. Therefore, several flavins, i.e., riboflavin, FMN and FAD, were tested to study direct interactions of flavins with nitroxides in the light, if any. There was no interaction between flavins and TEMPOL in aerobic solution as assayed by ESR. In anaerobic solution, TEMPOL signal loss was observed, but at a considerably slower rate than that observed for aerobic, aged SMP's. There was no effect of TEMPOL on FMN fluoroscence at TEMPOL concentrations that were tested (< 1 mM). Flavins photooxidize the reduced nitroxides TOLH and TAH. The photooxidation of TCH was also observed, but at a ten-fold lower rate than that of TOLH. To study the interaction between flavin radicals and nitroxides, FMN, 30 μM ^{14}N TOLH as an electron donor and 30 μM ^{15}N TEMPOL, as an electron acceptor, were illuminated anaerobically. The nitroxides having ^{14}N and ^{15}N paramagnetic centers give rise to distinct spectral

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lines; hence, reduction and oxidation rates can be monitored simultaneously. Upon illumination, there was a decrease in the ^{15}N TEMPOL signal and a simultaneous increase in the ^{14}N TEMPOL signal at the same rates. Upon admitting air to the sample under continuous illumination, both spectra increased to the original line height seen for 30 μ M ^{15}N TEMPOL.

Comparison of TEMPOL Reduction and Other Processes of Spin Loss: One-electron reduction of TEMPOL yields the non-paramagnetic hydroxylamine TOLH. Reoxidation of TOLH to TEMPOL is rapidly effected by ferricyanide. Thus, when 30 μ M of TOLH is treated with 2 mM potassium ferricyanide at pH 7.4, a spin signal rapidly appears, increasing to a maximum within two minutes at 25° C. The final line height corresponds to 30 μM of a fresh TEMPOL solution in the presence of 2 mM ferricyanide. Accordingly, ferricyanide oxidation was used in SMP's after illumination with TEMPOL to determine the fraction fo spin loss due to reduction. The remaining fraction of spin loss is operationally defined as nitroxide "destruction." If ferricyanide is added to SMP's during illumination, it effectively inhibits light-driven spin loss, i.e. spin loss rates progressively decrease with increasing ferricyanide concentrations such that in the presence of 7 μ M ferricyanide, the spin loss rate is half of what is observed in the absence of ferricyanide. Therefore, reoxidation of any TOLH produced during illumination was carried out in post-illumination experiments.

To compare reduction and destruction rates, illumination of 30 μ M TEMPOL and SMP's containing 10 mg/ml protein was conducted in an aerobic flask. At one-minute time intervals, 70 μ l samples were withdrawn for ESR analysis of TEMPOL line heights. 35 μl were examined directly and the remaining 35 μ l were treated with 2 mM ferricyanide. The signal heights were identical for the two sets of samples. A similar experiment was conducted with 100 M TOLH and SMP's. In this case, there was a light-driven appearance of TEMPOL line

heights in the samples. The ferricyanide treated samples showed that no spin destruction occurred initially. Spin destruction became apparent only after TOLH had been converted to TEMPOL during the illumination. When the TEMPOL concentration had reached 30μM, the destruction rate was comparable to what was observed for the sample containing only TEMPOL initially.

To study the effect of oxygen on spin loss, experiments were conducted in gas permeable tubing surrounded by air or nitrogen. Figure 3 shows that aerobic illumination of TEMPOL and SMP's causes less rapid spin loss than is observed under nitrogen. Upon admitting air into the anaerobic sample, a partial recovery of the spin signal is seen which gives way to complete signal loss upon prolonged illumination. This result is similar to aerobic illumination of TOLH, where transient and partial oxidation is observed under illumination. No oxidation of TOLH is seen under nitrogen. A comparison of initial rates seen for aerobic TEMPOL destruction and TOLH oxidation shows that destruction occurs at a rate comparable to oxidation.

Iron had a marked effect upon spin loss rates. As indicated earlier, ferricyanide was inhibitory at low concentrations. On the other hand, ferric chloride accelerated spin loss about two-fold at a concentration of 10μM. Considerable variability was seen in spin loss rates among different membrane preparations that were studied, possibly due to fluctuations in the concentrations and binding states of traces of iron among the different preparations.

The Role of Membranes in Spin Loss: To define the source of light-induced spin loss, SMP's that had been incubated in the dark for three hours at 37° C were centrifuged and spin destruction rates, i.e., aerobic rates of spin loss, were measured in the pellet, supernatant and in pellets that had been resuspended in their supernatants. The maximum spin loss rate was observed in the supernatant,

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the intermediate rate in the resuspended membranes and the minimum rate in the pellet. Washed pellets had no spin loss activity.

The effect of charged groups on the nitroxides is shown in the Table. The positively charged nitroxides exhibit significantly lower rates of spin loss and the positively charged hydroxylamine, TAH, a significantly higher photooxidation rate than the other nitroxides. The most hydrophobic nitroxide, TEMPO, exhibits the most rapid spin loss rate. The rates of photooxidation of the reduced nitroxides, i.e., TAH > TOLH > TCH is the same as observed for the direct interaction between flavins and reduced nitroxides. However, whereas TCH is photooxidized by FMN or riboflavin, only spin loss of a trace of TEMPCARBOXYLATE in the sample is observed with SMP's.

Flavin Fluorescence and Spin Loss Rates: Fig. 4 shows the aerobic spin loss rates and the appearance of flavin fluorescence in the supernatants of centrifuged SMP's as a function of time of incubation of the SMP's at 37° C in the dark. At two temperatures the two parameters increase in parallel with time up to about three hours of incubation. Longer incubation does not substantially increase flavin fluorescence or spin loss rates.

The Effect of Exogenous Flavins on Spin Loss Rates: SMP's that had been incubated in the dark at 37° C for various time periods were treated with FMN to study the effect of exogenous flavins on spin loss. As shown in Fig. 5, freshly isolated SMP's do not cause spin loss of nitroxides in the light even in the presence of added FMN. The presentation of data in this figure takes into account flavin release during dark incubation by referring to the results in Fig. 4. It is assumed tht the flavins observed in the supernatant of centrifuged SMP's behave photochemically like free FMN and hence can be pooled with exogenous flavins as "free flavins." Fig. 5 shows that at a fixed concentration of free flavin, there is an increase in aerobic spin loss rates with increasing time

of dark incubation. Similar data were obtained for SMP's that had been incubated in the dark under argon.

Partial Characterization of "Destroyed" TEMPOL: Radioactively labeled *TEMPOL was illuminated in the presence of SMP's for a sufficient period of time to completely destroy the spin. The suspension was centrifuged and essentially all of the radioactivity was found in the supernatant. Aliquots of the supernatant were adjusted to different pH values with phosphate buffer and partitioned against diethyl ether. The radioactivity of the ether extracts increased with pH such that about 10% of the total counts were observed at pH 10 while about 60% was seen at pH 12 in the ether fractions. Subsequently, another preparation of destroyed TEMPOL was extracted into ether at alkaline pH and the ether extract was oxidized with the strong oxidant m-chloroperoxybenzoic acid. The product of this procedure was concentrated and taken up in phosphate buffer at pH 7.4. An ESR spectrum of this sample was observed and was similar to a spectrum obtained when PRETOL was subjected to the same oxidation procedure.

Spin Trapping Experiments with Nitrones: In the presence of 100 mM DMPO, the rate of light-induced TEMPOL destruction with SMP's decreased by a factor of two, while 100 mM POBN completely inhibited TEMPOL destruction. An ESR spectrum was observed with DMPO both in the presence and absence of TEMPOL. This spectrum was identical to a spectrum obtained with DMPO and FMN in buffer (Fig. 6). No ESR spectrum was observed for POBN and SMP's with the broad-band interference filter. With white light a spectrum appeared very slowly, i.e., thirty minutes of illumination were required to obtain measureable coupling constants (A_N = 15.8 G, A_H = 2.0 G).

Relation of Spin Loss to Photodamage: Since freshly isolated SMP's exhibited little or no spin destruction with or without exogenous flavins and since aging of SMP's caused the appearance of the spin loss phenomenon

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in the aqueous environment of membranes, these diverse conditions were compared

in terms of photodamage to the respiratory chain and to membrane lipids.

Figure 7 shows that succinate oxidase activity is progressively lost under

illumination and that conditions which induce spin loss, i.e., addition

of supernatant from aged SMP's, only slightly accelerate photodamage of

these components of the respiratory chain. However, there is a marked

correlation between lipid peroxidation and spin destruction. The supernatant

treated SMP's yield considerably more TBA-reactive material during illumination

than either freshly isolated control SMP's or SMP's containing exogenous FMN

at a concentration equal to "released" flavin in the supernatant. Some aging

of the freshly isolated SMP's is reflected in the small spin loss rates seen

in the two preparations without added supernatant.

Photochemical Model Systems: EDTA is a well-known photoreductant for

flavins. Figure 8 shows that a TEMPOL signal was reduced on illuminating a

mixture of FMN and EDTA. Spin loss was rapid under nitrogen and could be

largely reversed when air was admitted to the sample. A sample that was

illuminated aerobically suffered relatively little spin loss.

DISCUSSION

Characterization of Nitroxide ESR Signal Changes: We were interested in using nitroxides as spin traps to elucidate photodamage mechanisms in mitochondria. Intact mitochondria were not suitable for study because their electron transport activity causes reduction of nitroxides in the dark (19). Therefore, our studies were conducted with isolated inner mitochondrial membranes (SMP's) where substrates for electron transport have been removed from the membranes. Since nitroxides are quite stable in the presence of SMP's in the dark, these preparations were deemed suitable for studies of photodamage with nitroxides.

Aerobic aged SMP's illuminated with visible light cause nitroxides to lose their paramagnetism and subsequent treatment of these SMP's with the oxidant ferricyanide does not cause a reappearance of the ESR signal; hence the phenomenon is not photoreduction of the nitroxides. It was also observed that the reduced nitroxides TOLH and TAH are photooxidized by aged SMP's and by flavins. By illuminating anaerobic SMP's and subsequently allowing them to become aerobic, a partial recovery of the nitroxide ESR signal was observed. Hence, under anaerobic conditions, some of the nitoxides are photoreduced to hydroxylamines under illumination. The use of gas permeable tubing permits a comparison of the effects of anaerobic and aerobic illumination in the same sample and thus represents a convenient tool for discriminating qualitatively between photoreduction and other mechanisms of spin loss. When this technique was applied to a simple photochemical system, FMN and EDTA, spin loss was seen to be comprised primarily of photoreduction. This is to be expected since FMN becomes photoreduced by EDTA anaerobically (20) and since it is known that reduced flavines reduce nitoxides (6).

The action spectrum of spin loss implicated flavins as the sensitizers for nitroxide spin loss. However, studies with simple model systems showed the

oxidized flavins had no effect on nitroxide spectra. Reduced flavins, FlH_2, and flavin radicals, $FlH\cdot$, reduce nitroxides (6). The experiment with ^{14}N TOLH and ^{15}N TEMPOL showed that neither the flavin radical $FlH\cdot$, which is necessarily produced during photooxidation of TOLH, nor its oxidation product with molecular oxygen cause spin destruction. Hence it is concluded that flavins are not directly responsible for the observed aerobic spin loss of nitroxides.

The association of spin loss activity with supernatants but not pellets of centrifuged SMP's implies that "released" flavins are necessary for the spin loss. "Released" flavins is an operational term since centrifugation speeds were insufficient to pellet individual proteins or membrane fragments. The most likely source of released flavin is NADH dehydrogenase, which contains non-covalently bound FMN. The steep temperature dependence of flavin release suggests that low concentrations of free flavins exist in SMP's illuminated at 10° C, where photoinactivation of respiratory activities is nevertheless observed (3).

Since certain amino acids are photoreductants for flavins (12,13), it seemed possible that anaerobic SMP's would cause nitroxide reduction under illumination. However, freshly isolated anaerobic SMP's exhibit no significant light-dependent spin reduction, even in the presence of $5 \mu M$ exogenous FMN or riboflavin (data not shown). Hence, mitochondrial membranes are poor photoreductants and direct oxidation of membranes, e.g., hydrogen abstraction from amino acid residues by free flavines is not likely to play an important role in' photodamage. The aerobic experiment with exogenous flavins (Fig. 5) showed that at a given concentration of free flavins there is an acceleration of nitroxide spin loss with aging of the membranes. Hence, some phenomenon other than flavin release occurs during the aging process which together with free flavins causes

spin loss. Taken together with the occurrence of spin loss in supernatants of centrifuged SMP's, it is inferred that some other factor(s) in addition to flavins are released from the SMP's into their aqueous environment during dark incubation. These factors react with excited flavins to bring about nitroxide spin loss. Since 30μM TEMPOL are destroyed by less than 2μM flavin, as estimated from supernatant fluorescence, it is clear the flavins act catalytically in the nitroxide destruction process, strengthening the conclusion that neither flavins nor their degradation products cause spin destruction.

Inference That Free Radicals Cause Nitroxide Destruction: Destruction of nitroxides to yield products not oxidizable by ferricyanide could occur by several mechanisms. One is the direct coupling of nitroxides, with other radicals. Known examples of radicals which form stable products with nitroxides include several alkyl radicals (4,5). Also, it has been shown in pulse radio-lysis experiments that nitroxides react with hydroxyl radicals (21). However, the products of the latter reaction seem to decompose rapidly since model systems, e.g., UV photolysis of H_2O_2 and Fenton reagents did not destroy the paramagnetic signal of TEMPOL. Another example of spin pairing is the reaction of nitroxides with thiyl radicals, a process which ultimately leads to the formation of tetramethyl piperidines, e.g., PRETOL (22). Studies of reactions between nitroxides and free radicals derived from ethylbenzene suggest that peroxy radicals, R'OO·, do not react with nitroxides (23). Oxidation of nitroxides with a powerful oxidant would yield immonium ions which would imme-diately decompose in aqueous solution to nitroxides and hydroxyl radicals (5). Under strongly reducing conditions, TEMPOL can be reduced to PRETOL. This mechanism cannot explain the spin loss observed in SSMP's, however, since no destruction occurs with TOLH. Thus, the mechanism of spin destruction appears

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to be radical pairing of nitroxides with other radicals, excluding oxygen-centered radicals, like hydroxyl or peroxy radicals.

The partial characterization of the ultimate reaction product of TEMPOL in illuminated aerobic SMP's, particularly the high pK_a value, suggests formation of PRETOL. A similar reaction is the formation of piperidines in the reaction of thiyl radicals with nitroxides (22). This is not necessarily the reaction mechanism in illuminated SMP's, however. In model system studies with alkyl thiols and flavins, no significant rate of light-induced nitroxide destruction was observed.

Nitrone spin traps like DMPO and POBN have been used extensively to demonstrate the existence of free radicals (24). Since the ESR spectrum arising from DMPO with illuminated SMP's is identical to the spectrum seen with only DMPO and FMN, there appears to be no correlation between nitroxide spin destruction and DMPO spin formation. It was suggested previously that nitroxides could arise from DMPO and light-excited flavins by a non-radical mechanism (25). The spin adduct seen with POBN with white light is difficult to interpret owing to its rather low rate of appearance and the failure to observe an adduct with the broad-band interference filter. In summary, these nitrone traps do not appear to trap radicals in illuminated SMP's even under conditions of rapid TEMPOL destruction. This does not rule out the interpretation that TEMPOL destruction is caused by radicals; rather it suggests that these nitrones with illuminated SMP's do not form radicals sufficiently stable to be observed by ESR spectrometry.

Relationship to Membrane Damage: It was of interest to determine whether the free radicals trapped with nitroxides played a role in the mechanism of flavin-sensitized photodamage. In freshly isolated SMP's without exogenous FMN, there is appreciable photodmage to succinate oxidase even though no radicals were detected under these conditions with TEMPOL. Thus, it is inferred

that bound flavin exerts its destructive effects in close proximity to its binding site, and that there is no propagation of damage to distant sites as seen in the lack of lipid peroxidation and spin destruction. Exogenous FMN increases photodamage only slightly, suggesting that singlet oxygen generated by free flavin is not a major damaging species in SMP photodamage.

The addition of supernatant from aged SMP's greatly increases lipid peroxidation and slightly increases inactivation of succinate oxidase. Since the supernatant contains factor(s) that interact with light-excited flavins to produce free radicals, it is seen that the resulting radicals have destructive potential, including the initiation of lipid peroxidation and enzyme destruction. Once initiated by exogenous radicals, free radical propagation does seem to occur in the SMP membranes since the most hydrophobic nitroxide, TEMPO, is destroyed appreciably more rapidly than the other probes. The relevance of these observations to photodamage in vivo is not clear. The factors we describe in aged SMP's which form radicals upon reacting with excited free flavins could also be present in vivo. Since they are water soluble, they would be removed from the SMP's during membrane isolation procedures, and hence freshly centrifuged SMP's would not exhibit light-dependent nitroxide destruction. Given the potential of these free-radical precursors for causing damage to lipids and enzymes, their further characterization is certainly of interest.

ACKNOWLEDGMENTS

REFERENCES

1. B.L. Epel (1973) Photophys. $\underline{8}$, 209-229.

2. B.B. Aggarwal (1977) Ph.D. Thesis, University of California, Berkeley, Lawrence Berkeley Laboratory Report LBL-6848.

3. B.B. Aggarwal, A.T. Quintanilha, R. Cammack and L. Packer (1978) Biochim. Biophys. Acta $\underline{502}$, 367-382.

4. J.F.W. Keana, R.J. Dinerstein and F. Baitis (1971) J. Org. Chem. $\underline{36}$, 209-211.

5. E.G. Rozantzev (1970) Free Nitroxyl Radicals, Plenum Press, New York, London.

6. T.W. Chan and T.C. Bruice (1977) J. Am. Chem. Soc. $\underline{99}$, 7287-7291.

7. A.T. Quintanilha and L. Packer (1977) Proc. Natl. Acad. Sci. U.S. $\underline{74}$, 570-576.

8. E.J. Rauckman, G.M. Rosen and B.B. Kitchell (1978) Mol. Pharmacol. $\underline{15}$, 131-137.

9. A. Rigo, E. Argese, R. Stevanato, E.F. Orsega and P. Viglino (1977) Inorg. Chim. Acta $\underline{24}$, L71-L73.

10. Y. Lion, M. Delmelle, A. Van de Vorst (1976) Nature $\underline{263}$, 442-443.

11. Foote, C.S. (1976) in Free Radicals in Biology, Vol. II, W.A. Pryor, ed. Academic Press, New York, pp. 85-133.

12. Penzer, G.R. and G.K. Radda (1968) Biochem. J. $\underline{109}$, 259-268.

13. Penzer, G.R. (1970) Biochem. J. $\underline{116}$, 733-743.

14. Tu, S.-C. and D.B. McCormick (1973) J. Biol. Chem. $\underline{18}$, 6339-6347.

15. Massey, V., M. Stankovich, and P. Hemmerich (1978) Biochemistry $\underline{17}$, 1-8.

16. E.J. Rauckman, G.M. Rosen and M.B. Abou-Donia (1976) J. Org. Chem.. $\underline{41}$, 564-565.

17. R.J. Mehlhorn and L. Packer (1976) Biochim. Biophys. Acta $\underline{423}$, 382-397..

18. Plachy, W.Z. and D.A. Windrem (1977) J. Mag. Resonance $\underline{27}$, 237-239.

19. Quintanilha, A.T. and L. Packer (1977) Proc. Natl. Acad. Sci. U.S.A. $\underline{74}$, 570-576.

20. L.P. Vernon (1959) Biochim. Biophys. Acta $\underline{36}$, 177-185.

21. Asmus, K.-D., S. Nigam and R.L. Willson (1976) Int. J. Radiat. Biol.

29, 211-219.

22. Murayama, K. and Yoshioka, T. (1969) Bull. Chem. Soc. Japan 42, 1942-1947.

23. Neiman, M.B. and E.G. Rozantzev (1966) Izv. Akad. Nauk. SSR, Ser. Khim. 1966, 571.

24. Janzen, E.G. (1980) in Free Radicals in Biology, Vol. IV, W.A. Pryor, ed. Academic Press, New York, pp. 115-154.

25. Finkelstein, E., G.M. Rosen and E.J. Rauckman (1980) J. Am. Chem. Soc. 102, 4994-4999.

TABLE

Light-dependent aerobic spin loss and oxidation rates for different nitroxides and hydroxylamines. Protein, 10 mg/ml, SMP's preincubated 2 hours at 37° C in the dark.

Nitroxide	TEMPO	TEMPOL	TEMP-AMINE	CAT_1	TEMP-CARBOX-YLATE	TEMP-SUL-FATE
Initial Rate μM/min	32	16	5.5	3.8	11.5	9.5

HYDROXYLAMINE	TOLH	TAH	TOH
Initial Rate $-\mu$M/min	3.1	12.7	-2.8

FIGURE LEGENDS

Figure 1. The line height of TEMPOL expressed in terms of known TEMPOL con-
centration in buffer as a function of illumination. Inset, ESR
spectrum of TEMPOL in SMP; arrow points to the field position where
the line height was monitored. Upward arrows denote light on,
downward light off. Protein concentration 12 mg/ml. SMP incubated
for three hours at 36° C prior to white light illumination.

Figure 2. Action spectrum of spin loss rates. SMP at 29 mg/ml protein,
were incubated for 1.5 hrs at 37° C prior to illumination. Two
experiments are denoted by ● and ▲. The solid line shows an ab-
sorption spectrum of 40 M FMN, the absorbance units are arbitrary.

Figure 3. Aerobic and anaerobic ESR line height changes of TEMPOL in illumi-
nated SMP's. Conditions as in Fig. 1. A) 30μM TEMPOL in air, B)
30μM TEMPOL under N_2, C) 30μM TOLH in air, D) same sample as
B, but now in air.

Figure 4. SMP supernatant flavin fluorescence and aerobic TEMPOL spin loss
rates as a function of incubation time in the dark. SMP protein
was 7.9 mg/ml. △ : Supernatant flavin fluorescence at 31° C,
O: Spin loss rate at 31° C, ■: Supernatant flavin fluorescence
at 37° C, ◆: Spin loss rate at 37° C. Flavin fluorescence is
expressed as FMN equivalents.

Figure 5:   Effect of dark incubation time and exogenous FMN on aerobic TEMPOL
            spin loss rates.   SMP protein was 10 mg/ml.   Dashed lines indicate
            that the free flavin concentration for that region of the curve was
            estimated from the flavin supernatant fluorescence data in Fig.
            4.

Figure 6:   A)   Spectrum of DMPO adducts in SMP's.   SMP protein was 10.8 mg/ml;
            DMPO concentration was 100 mM.   B)   Spectrum formed within two
            minutes of illumination of 100 mM DMPO and 10μM FMN in the cavity
            of the ESR instrument.

Figure 7:   Effect of addition of FMN or supernatant from aged SMP's on spin
            loss rates (A), light dependent succinate oxidase inactivation (B),
            formation of TBA-reactive materials (C).   SMP's at 12 mg/ml protein.
            (——) control, (– – –) 2.7μM FMN, (—·—·) supernatant.

Figure 8:   Aerobic and anaerobic ESR line height changes of TEMPOL and TOLH
            in a photochemical model system.   The model system contained 10μM
            FMN, 10 mM EDTA, and 30 mM potassium phosphate, pH 7.4.   Aerobic
            illumination, and alternately anaerobic and aerobic illumination
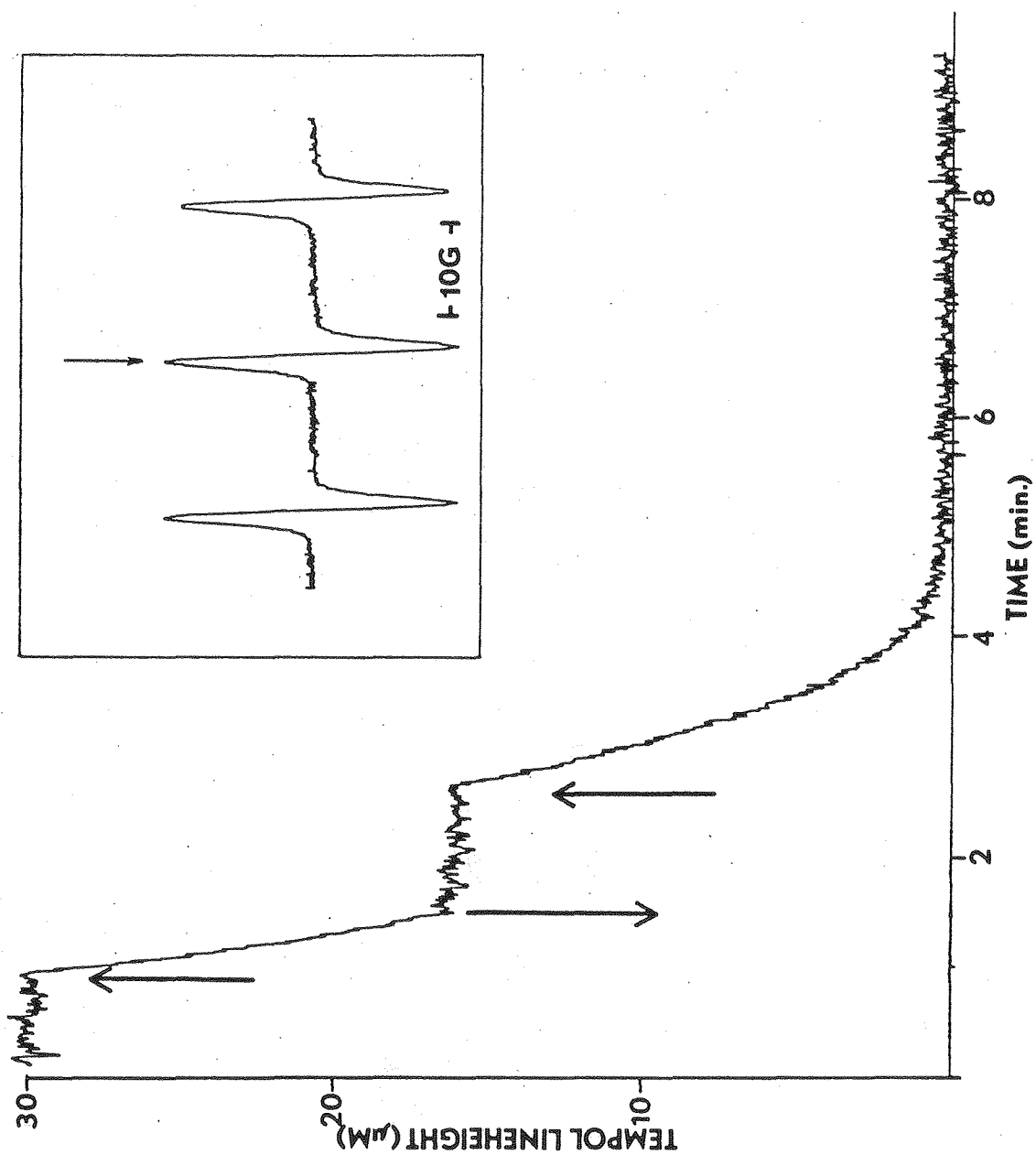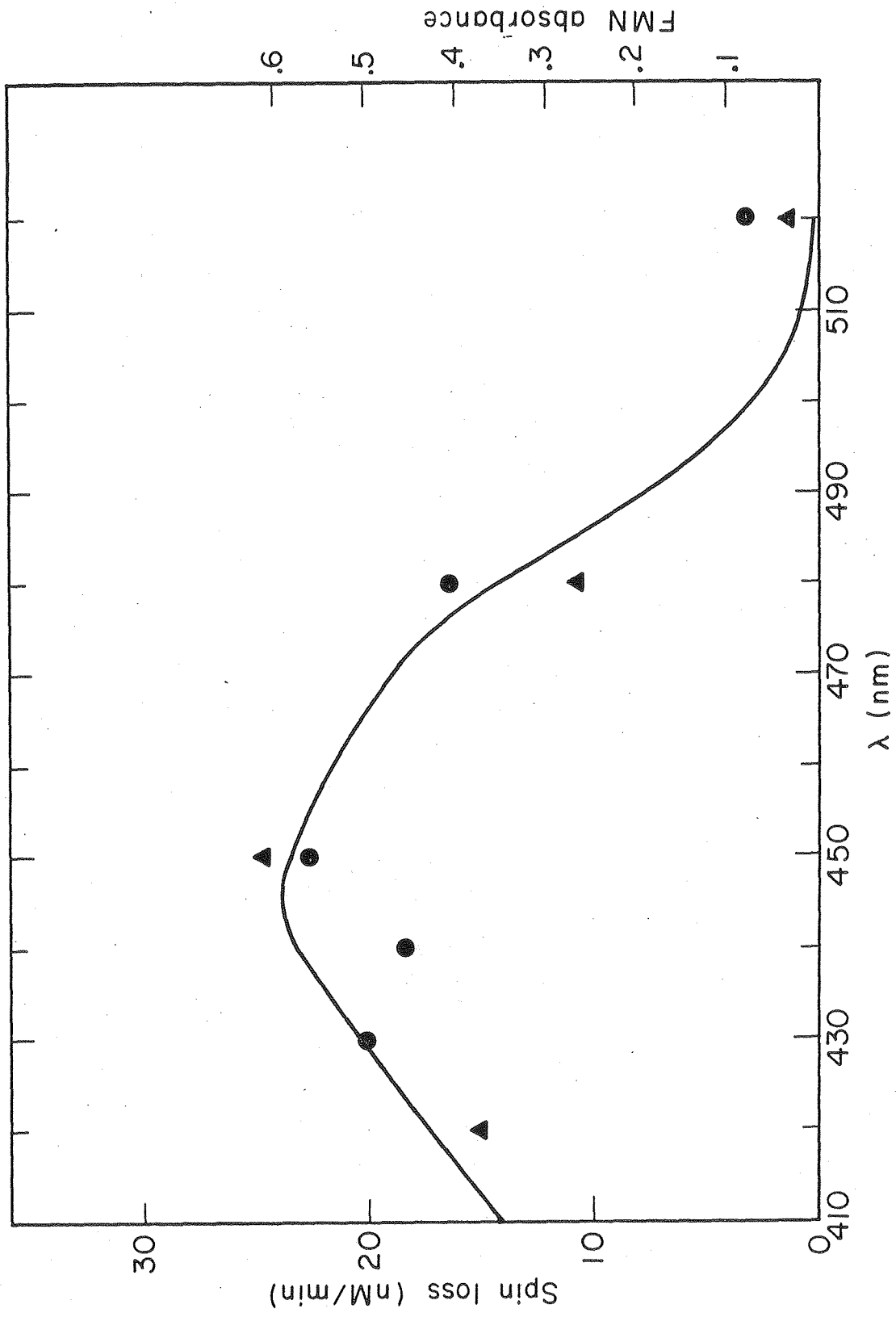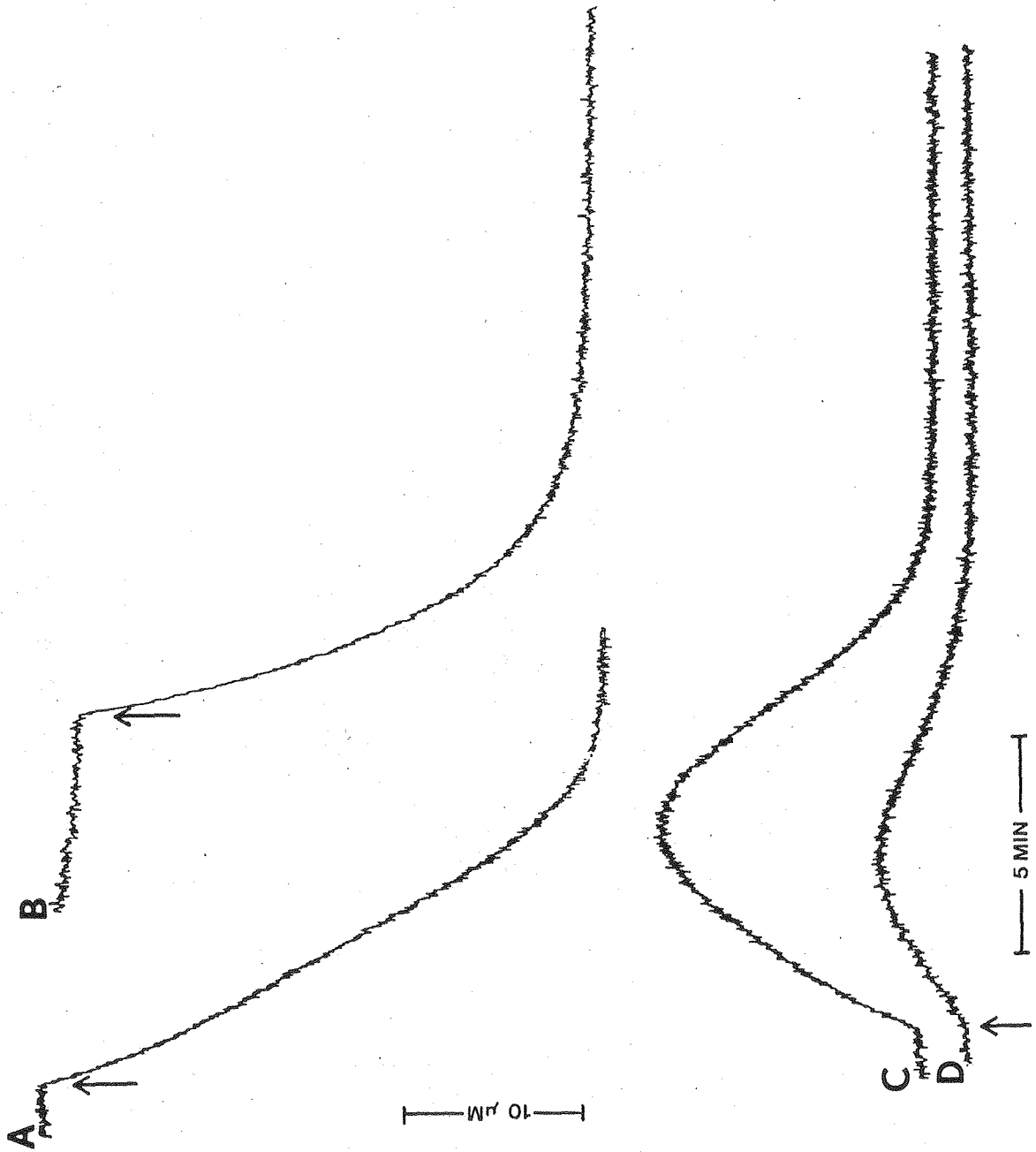   as indicated.

TEMPOL LINEHEIGHT (μM)

TIME (min.)

Figure 1

Figure 2

XBL7710-3877

Figure 3
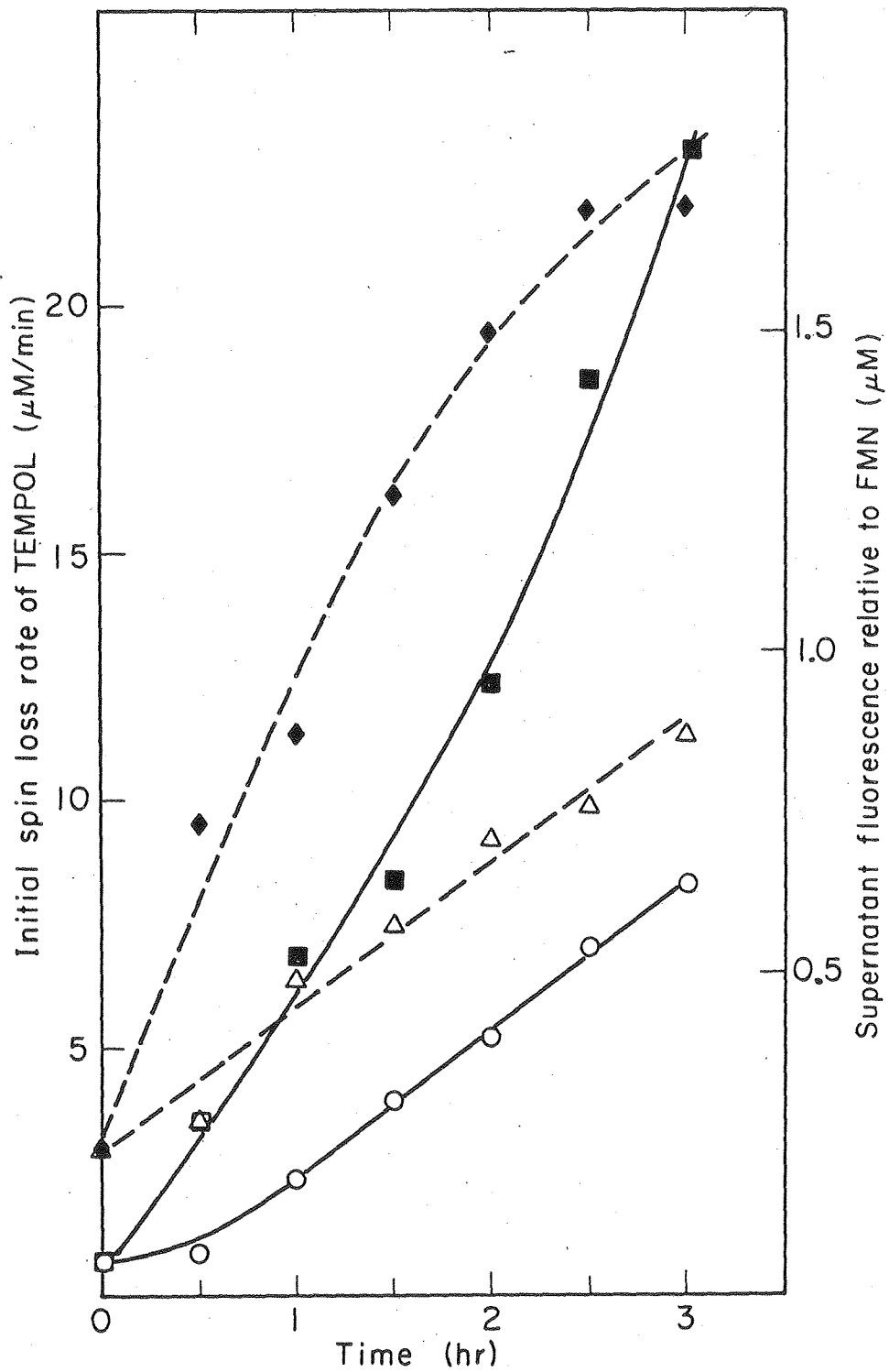
Figure 4

XBL8011-3864

XBL8012-3893

Fiugre 5

Figure 6

20 G

A

B

30

XBL 815-9489

Figure 7

TEMPOL, AIR

TEMPOL
N₂

TOLH, AIR

AIR

XBL80ll-3865

Figure 8