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AUTOXIDATION AND CYTOTOXICITY *

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We essay the presentation of a comprehensive synthesis, or reaction schema, to relate autoxidations of non-lipid compounds to lipid chain peroxidation *in vivo*. As at Pinewa three years ago, we do this in the context of cytotoxic autoxidation reactions, and now--as then (1)-- we conclude that hydroxyl radicals, $OH^{\cdot}$, produced by iron-dependent Fenton reactions serve as both primary toxicants and as sources of secondary toxicants. The latter stem from lipid chain peroxidation initiated by the Fenton-derived $OH^{\cdot}$, which we visualize as the obligate coupling step linking enzyme-dependent and non-enzymic autoxidations to potentially toxic outcomes.

HYDROXYL RADICALS AND MEMBRANE LIPID PEROXIDATION

At Pinewa we cited evidence implicating the generation of $OH^{\cdot}$ in "dark" biochemistry (i.e., *not* photochemistry or radiation chemistry) (1), and a surge of new data in support of this contention has appeared in the intervening few years, as recently reviewed by Bors, Saran and Czapski (see Table III of ref. 2). We proposed (1) that in addition to the recognized radiomimetic cytotoxicity of the extremely reactive (and, hence, short-lived and short-ranged) hydroxyl radicals themselves, their initiation of lipid peroxidation in membrane lipids could slowly (hours) "broadcast" damage to important ("target") biomolecules at considerable distances, mediated by reactive intermediates and products of

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lipid chain peroxidation, both free radical (e.g., lipid alkoxy radicals) and non-radical (e.g., carbonyl compounds (3)) in nature.

AN INTEGRATED REACTION SCHEMA BASED ON AUTOXIDATION

A reaction schema justified by the arguments reviewed in detail in our previous paper (1) is depicted by Fig. 1. In the figure, electron flow (i.e., the transfer of redox equivalents) from the critical autoxidation step (beaded arrow) through the Fenton-like iron cycle to yield $OH^{\cdot}$ reacting with [membrane] lipid, LH, is emphasized by the set of heavy arrows.

Although the capability of $HO_2^{\cdot}/O_2^{\cdot-}$ directly to reinitiate lipid peroxidation chains from metastable lipid hydroperoxides, LOOH, (the reaction denoted by a question mark in Fig. 1) has been challenged by contrary findings (4,5), and Winterbourn has claimed that the reduction of ferric iron by ascorbate (looping arrow given by long dashes and connecting the ultimate electron donor, DH_2, with complexed iron, [Fe--], in Fig. 1) is faster than its reaction with $O_2^{\cdot-}$ and probably more important in vivo (6), the schema implied by our earlier paper remains otherwise intact (Fig. 1).

The critical feature in driving the cycling iron-dependent generation of $OH^{\cdot}$ or of lipid alkoxy radicals ($LO^{\cdot}$) is the provision of an autoxidizing substrate that reacts spontaneously with dioxygen to yield $O_2^{\cdot-}/HO^{\cdot}_2$ (beaded arrow in Fig. 1). The basic schema of Fig. 1 relates this autoxidation to the linked reactions of quinol-type cytotoxins which catalyze electron donation via an iron redox cycle from ascorbate or other ultimate reductants to H_2O_2 or to LOOH, because autoxidation reactions of those quinols were the focus of our concern at Pinewa. The H_2O_2, in turn, can be provided endogenously by spontaneous or dismutase-catalyzed disproportionation of the superoxide afforded by the autoxidation step, Fig. 1. However, we emphasize

here other versions of the schema that are conceptually equivalent and differ only in the way that the autoxidizing substrate is provided.

In the cases of cytotoxins such as 6-hydroxydopamine, 6-aminodopamine and their congeners or of alloxan (which is reversibly reduced to dialuric acid *in vivo*), we contended that it was their quinolic forms (or quinolamine equivalents) which were the active substrates for autoxidation (1). The reactions summarized and integrated by the cycles to the right of the autoxidation reaction (beaded arrow) in the basic schema of Fig. 1 were invoked to explain the potentiation of the cytotoxicity of these agents *in vivo* by ascorbic acid, their catalysis of ascorbate oxidation *in vitro* (7), and certain other observations relating to these interactions (see Charts 1 and 2 of ref. 1). However, in cases where electron affinic drugs (such as nitrofurans, nitrobenzenes, etc.) or carcinogens (such as 4-nitroquinoline-1-oxide, 4-nitropyridine-1-oxide, etc.) catalyze the oxidation of ascorbate (8) or interact with non-protein thiols in cells (9), it appears that spontaneous reduction of these compounds by ascorbate can occur to afford nitro radical anions as reactive species which can also autoxidize in the presence of dioxygen (8,10). A version of the reaction schema that incorporates these observations and affords semiquinones rather than quinols (hydroquinones) as the autoxidizable substrates in non-enzymic reactions is given by Fig. 1A.

SCHEMA FOR AUTOXIDATION DEPENDING UPON ENZYMIC REDUCTION

When the reduction of ultimate electron donor to autoxidation substrate is enzymic *in vitro* or *in vivo*, the schema of Fig. 1B applies. Moreover, this generalized pathway appears consistent with many biochemical and metabolic findings regarding the detection of $O_2^{\cdot-}$, H_2O_2, or $OH^{\cdot}$ (the latter directly by spin-trapping or inferentially by product analysis) or of products associated with lipid peroxidation from metabolizing or NAD(P)H-supported cells, microsomes,

and solubilized microsomal enzyme systems. Recent findings (1) show that even nuclei and nuclear membrane preparations undergo iron-stimulated, NADPH-supported lipid peroxidation.

In general, any compound that can enter cells and accept electrons from an appropriate carrier to give rise to an autoxidizable form could be expected to enter into a reaction schema like that in Fig. 1B, producing $O_2^{\cdot-}$ and H_2O_2 (12) and--as an ultimate toxin--perhaps $OH^{\cdot}$. Indeed, substances such as paraquat, methyl viologen, etc. (12) and various nitro (10,13) and azo compounds (14) do increase intracellular production of $O_2^{\cdot-}$ and H_2O_2, and the toxicity of some of them increases as pO_2 rises (12).

Experimental evidence suggests that within cells most of the autoxidizable intermediates formed by enzymic action are associated with microsomal oxidases. There is some indication that reduced forms of microsomal cytochrome reductases (15,16) and certain valence states of P-450-type cytochromes themselves (J.A. Peterson, this conference) are autoxidizable and that P-450 cytochromes may directly effect one-electron reduction of some substrates (most substrates are oxidized or hydroxylated). However, the weight of evidence appears to favor the direct enzymic reduction of substrates to autoxidizable products by the flavoprotein cytochrome reductases associated with the microsomal and mitochondrial cytochrome P-450 oxidase/monoxygenase systems.

ROLE OF FLAVOPROTEIN REDUCTASES

In support of electron flow from reduced pyridine nucleotides through flavoprotein cytochrome reductases to compounds that are thence converted into autoxidation substrates are observations that solubilized, purified preparations of microsomal reductase will support $OH^{\cdot}$ generation (detected by spin-trapping) and lipid peroxidation (malonaldehyde production) when stimulated by chelated iron (Fe^{2+}-EDTA) and provided reducing equivalents by NADPH (17). Comparable

behavior of intact rat liver microsomes also appears to be independent of cytochrome P-450 when the latter is inhibited by metyrapone (16,18). In a similar vein, rat liver cytochrome P-450 reductase without the oxidase present catalyzes reduction by NADPH of tumoricidal quinone anthracycline antibiotics to rapidly autoxidizable anion free radicals (19). Also indicative of the competence of flavocompounds alone to mediate NADPH-supported reduction of substrates to autoxidizable products is the report that a model system of nitro compounds, NAD(P)H, and flavin mononucleotide (FMN) can form autoxidizable nitro dianion free radicals with no heme present (15).

As another line of evidence supporting the participation of flavoproteins, hepatic microsomal lipid peroxidation is itself independent of cytochrome P-450, and even when the lipid oxidation is inhibited by drug substrates of the cytochrome P-450 mixed function oxidase in intact microsomes, the inhibition fails to correlate with drug metabolism. Probably, this is so because direct suppression of lipid chain peroxidation results from the antioxidant properties of the drug substrates and does not reflect competition between drug metabolism by the oxidase and lipid peroxidation for NADPH reducing equivalents (20).

The oxidation of ethanol by rat liver microsomes is also dissociated from drug metabolism, and it is yet another metabolic process that depends on OH· mediation. Microsomal oxidation of alcohol is not correlated with mixed function oxidation of drug substrates in intact microsomes and even proceeds in the absence of cytochrome P-450 in reconstituted systems (21). Rather than depending on cytochrome oxidation, the microsomal production of OH· from endogenous H_2O_2 and the subsequent oxidation of primary aliphatic alcohols reveals patterns of product yields and of inhibition consistent with a Fenton system (21,22).

Additional support for the nonessentiality of cytochrome P-450 for enzyme-generated autoxidation in cells comes from detergent-solubilized superoxide-

forming enzyme from human polymorphonuclear leukocytes (PMN's), which is associated with the well-known elaboration of $O_2^{\cdot-}$ and associated reactive forms of oxygen that accompany the bactericidal and cytocidal components of the "oxidative burst" of activated PMN's and macrophages. This purified enzyme requires only NADPH and a flavin (flavin adenine dinucleotide is best) for its activity, with no need for cytochrome P-450 (23).

ADEQUACY OF FENTON-SUPPORTED INITIATION OF LIPID PEROXIDATION

Although we argue that Fenton-supported lipid peroxidation can be mediated by OH·, as our schema shows (Fig. 1), Aust and his colleagues hold that Fenton-generated OH· is incapable of initiating lipid peroxidation in biological membranes and that "perferryl" iron-oxygen complexes, such as those alleged to form with iron chelated by ADP, are the obligate "primordial" initiators of lipid chain peroxidation therein (24,25). Above and beyond our disagreement concerning the nomenclature (see discussion, these proceedings), we remain unconvinced. We cannot readily fit a cycling reaction involving the implied iron-oxy complex, $Fe(II)\text{-}O_2 \leftrightarrow Fe(III)\text{-}O_2^{\cdot-}$, into our integrated reaction schema, but what is far more compelling is that we note that Fenton-type reactions in homogeneous reaction systems seem largely oblivious of whether ADP is present or not, even when lipid peroxidation is involved, whereas most--or all--of the systems that fail to follow expected Fenton behavior are heterogeneous, multiphasic ones with some compartmentalized and membrane-bound reactants.

With lipids in membranes or vesicles, such as the microvesicular extracted microsomal lipids used by Aust et al. (24,25), the rate constants for the reaction of OH· with lipid may be lowered by nearly an order of magnitude (26,27). Hence, even Fenton initiation would be expected to be less efficient than with solubilized lipids. Considering the short diffusion

range of reactive OH· radicals, we have the notion that ADP may serve to distribute the redox-active iron so as to increase OH· concentrations locally when peroxidation of vesicular lipids is initiated *in vitro* by Fenton reactions in the suspending medium. OH· production *in vivo* at cell surfaces or intracellularly, therefore, may represent an altogether different situation.

Failure of inhibitor patterns in biological, compartmentalized systems to follow expectations based upon Fenton reactions in solution (especially ineffectiveness of catalase in some cases (28,29) may also be explained by a speculation of Green *et al.* (29). They propose that within cells H_2O_2 may be inaccessible to catalase if the H_2O_2 remains coordinated to a metal ion (represented by brackets in the equations below). Then OH· would appear to be formed directly from $O_2^{\cdot-}$ by a disproportionation reaction due to the set of reactions occurring within the locus of the reducing metal (such as Fe^{2+}), and no "free" H_2O_2 would exist:

$$H^+ + O_2^{\cdot-} \longrightarrow HO_2^{\cdot}$$

$$HO_2^{\cdot} + H^+ + O_2^{\cdot-} \longrightarrow [H_2O_2] + O_2$$

$$[Fe^{2+}] + [H_2O_2] \longrightarrow OH\cdot + [Fe^{3+}] + OH^-$$

Net: $2O_2^{\cdot-} + [Fe^{2+}] + 2H^+ \text{ --- } OH\cdot + OH^- + O_2 + [Fe^{3+}]$.

Contrary to Aust's claims, with peroxidation of lipids in intact microsomes OH· does appear to be present, because spin trapping reveals authentic OH· adducts (16,18). Furthermore, as we asserted above and document below, Fenton-generated OH· seems to be an effective initiator of lipid peroxidation in homogeneous reactions, and the presence of ADP makes little difference (provided that iron is otherwise complexed to avoid precipitation). Even in the presence of ADP the relatively "hydroxyl-specific" spin trap,

4-pyridyl-1-oxide-N-tertbutyl nitrone, forms its characteristic $OH^{\cdot}$ adduct in a Fenton reaction (30). As another example: reducing agent inhibition of Fenton-catalyzed linoleic acid peroxidation is no different whether Fe^{2+} that contaminates the buffer is used, additional $FeSO_4$ is provided, or ADP-Fe^{2+} is added (20).

Even enzymically-induced lipid oxidation can be initiated by $OH^{\cdot}$ in homogeneous reaction systems. Thus with detergent-solubilized methyl linoleate, cytochrome P-450 reductase, NADPH, and $FeCl_3$ (even when excess EDTA is present), lipid peroxidation occurs and reveals an inhibitor pattern expected of a Fenton system with $OH^{\cdot}$ intermediation (17,31).

Our own studies also support the concept of $OH^{\cdot}$ initiation of lipid oxidation in Fenton-like reactions. Previously we reported a pattern of oxygen consumption with 6-hydroxydopamine or 6-aminodopamine catalysis of iron-enhanced oxidation of ascorbate in the presence of arachidonic acid that was interpreted as most consistent with $OH^{\cdot}$ initiation of lipid oxidation (1). Fig. 2 reveals that with Fe^{2+} and an excess of ascorbate to keep the iron reduced and to provide net reducing power (as in Fig. 1), there is a background rate of O_2 consumption by metal-catalyzed ascorbate autoxidation. Addition of exogenous H_2O_2 to complete a Fenton system has little further effect except in the presence of linoleic acid, which increases O_2 consumption. The additional O_2 consumption, in turn, is taken to be a measure of peroxy radical formation (see $LOO^{\cdot}$ in Fig. 1) as the lipid alkyl radicals, $L^{\cdot}$, produced by reaction with $OH^{\cdot}$ rapidly react with O_2 (32). The addition of ADP does not alter this pattern of O_2 consumption (Fig. 2), regardless of whether ADP is present before Fe^{2+} is added, as in Fig. 2, or whether ADP and Fe^{2+} are premixed anaerobically.

Furthermore, Fig. 2 represents a more generalized behavior, because linoleic acid can be replaced by linolenic acid, and enhanced O_2 consumption from lipid oxidation cum peroxidation is also seen when Ti^{3+} is substituted for iron/ascorbate as the Fenton reductant. Iron sequestration quenches O_2 consumption, as expected, and $OH^{\cdot}$ scavengers (including the spin trap 4-hydroxyphenyl-N-tertbutyl nitrone) eliminate the enhanced O_2 uptake that is due to the reaction of Fenton $OH^{\cdot}$ with lipid (work in progress).

In summary, we conclude that our proposed schema provides a useful integration into a concerted toxicological pathway of various cellular and laboratory redox processes that have been studied as isolated reaction systems. Guided by the schema, which will require revision as new data appear, we may now begin to study cellular cytotoxic processes for evidence that the connected reactions actually occur in vivo.

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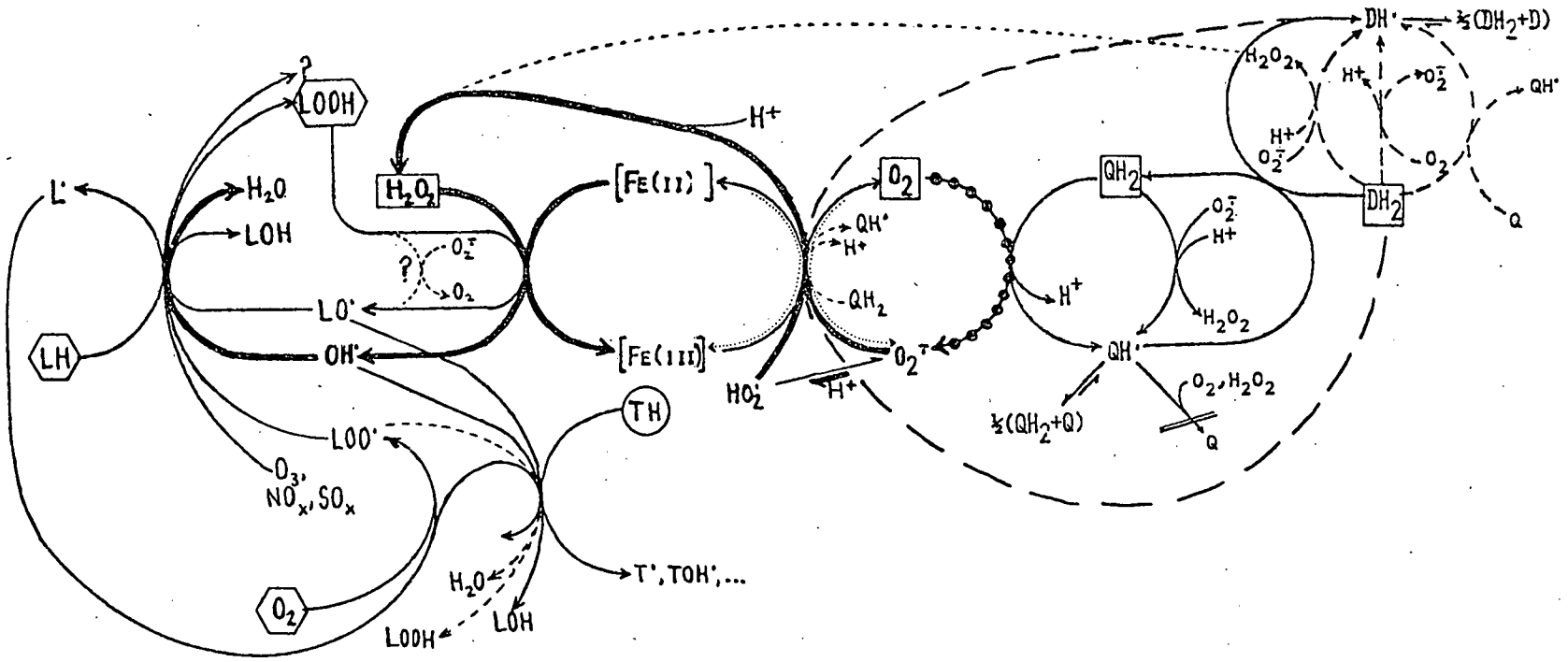
LEGENDS FOR FIGURES

Fig. 1 Iron-dependent, biological autoxidation schema. Non-lipid substrates: When autoxidation catalyst is present, DH_2 = ultimate electron donor (ascorbate anion, etc.) in its hydroquinone (quinol) form, with $DH^{\cdot}$ its free radical (semiquinone) form and D its quinone form; $QH_2(QH^{\cdot},Q)$ = autoxidation catalyst or ultimate electron donor in the absence of DH_2; TH = target ("essential") biomolecule; [Fe--] = iron complexed to ADP, ascorbate, EDTA, etc. in vitro and to undetermined ligands in vivo. Lipid substrates: LH = polyunsaturated lipid; $L^{\cdot}$ = lipid alkyl radical; $LO^{\cdot}$ = lipid alkoxy radical; $LOO^{\cdot}$ = lipid peroxy radical; LOOH = lipid hydroperoxide.

Fig. 2 Oxygen consumption at 37° C and pH 7.4 (0.1 M phosphate buffer). Arrows indicate times of addition of various reactants in 10-30 µl aliquots into 3 ml total volume. Final concentrations: Adenosine diphosphate (ADP) = 0.2 mM; Ascorbate = 3.3 mM; Fe(II), as $Fe(NH_4)_2(SO_4)_2$ = 0.1 mM; Acetonitrile (AN) = 67 mM; Linoleic acid in AN = 0.3 mM; H_2O_2 = 0.3 mM. Tracings have been normalized (superimposed) at the point where H_2O_2 is added to complete the Fenton reaction system. The dashed line (---) represents O_2 consumption in the absence of lipid. For the other two tracings linoleic acid was added before the Fenton reaction commenced, either with (solid line, ——) or without (dotted line, ...) ADP present.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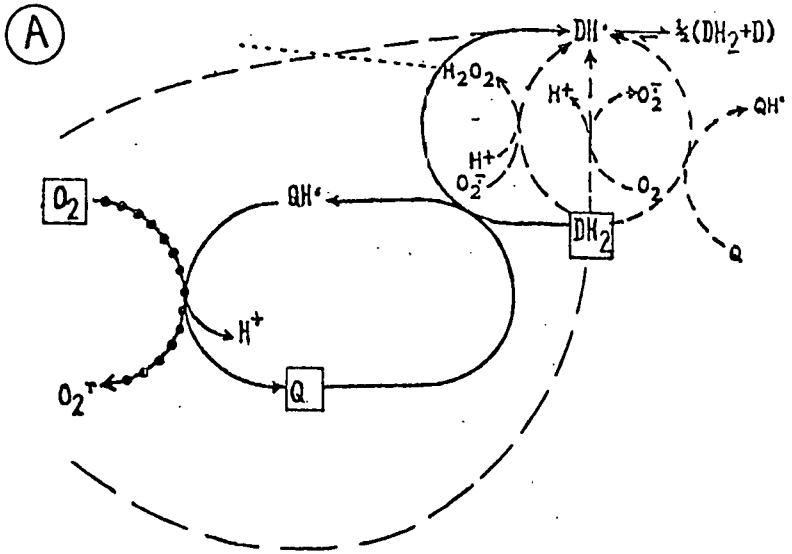
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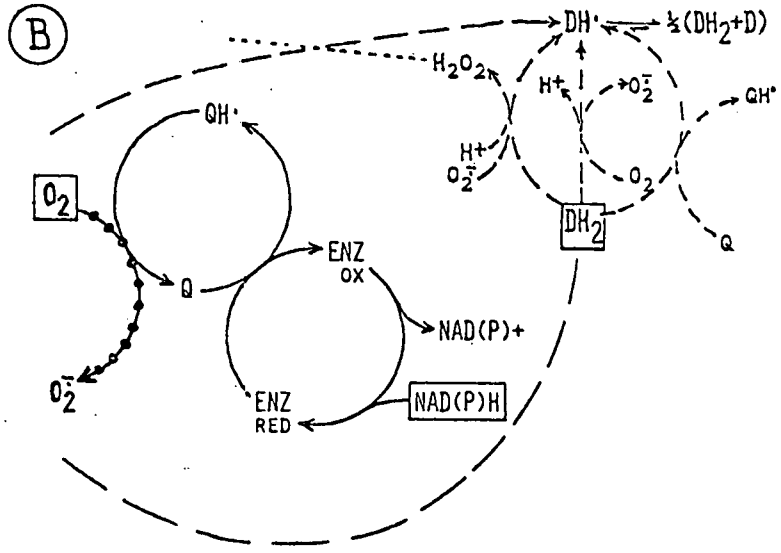
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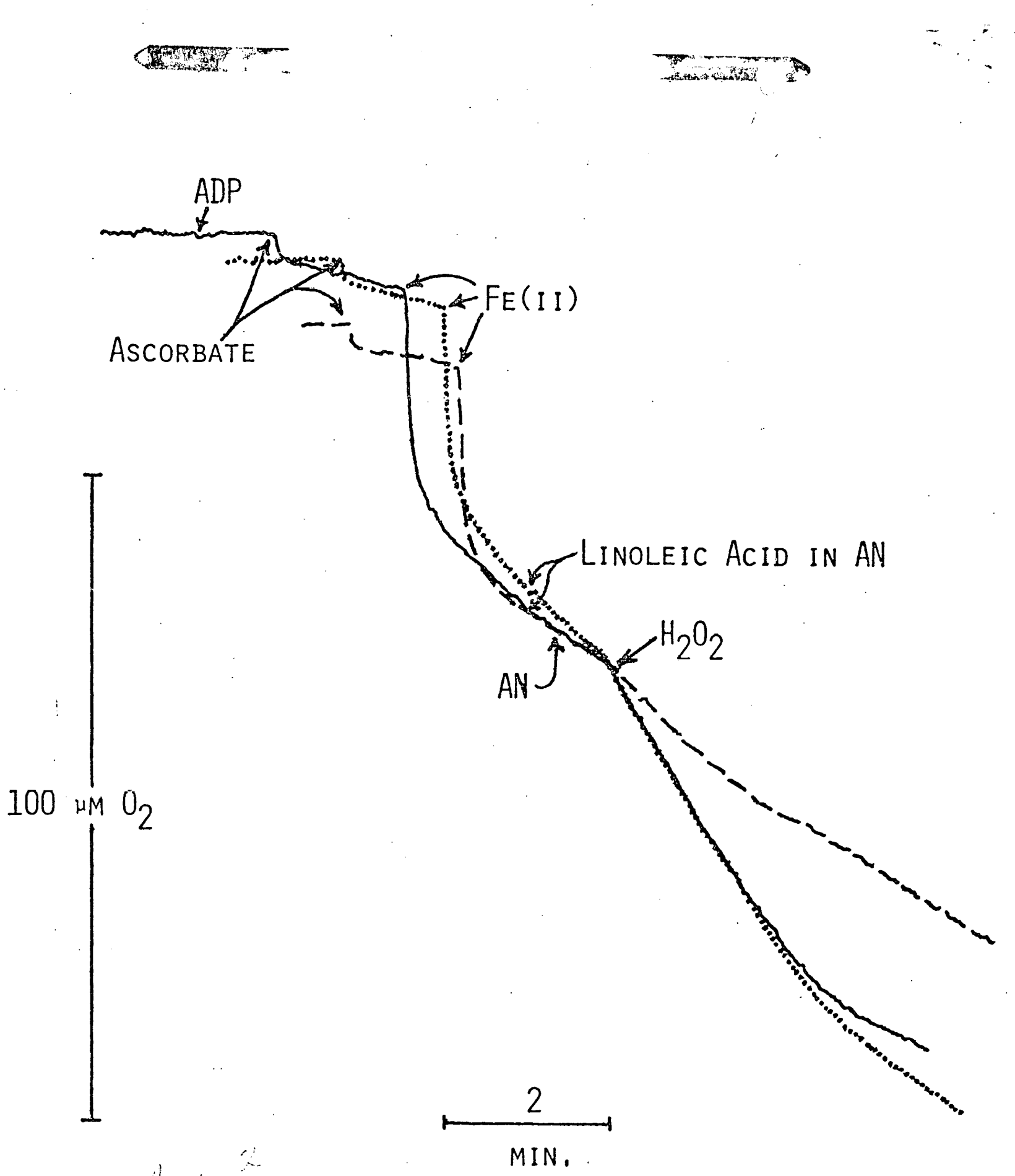
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