STUDIES ON OXIDATIVE PHOSPHORYLATION:

OXIDATION OF QUINOL PHOSPHATES

by

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Dedication

To my wife whose endurance and patience made this possible.

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1. STATEMENT OF PROBLEM

Any definition of life must imply the existence of highly ordered, relatively low entropy systems. Even the simplest organisms, therefore, are thermodynamically improbable. Furthermore, the series of dynamic chemical reactions responsible for organic function is not at equilibrium nor may it attain equilibrium except through death (48). Consequently, there is a continuous conversion of free energy to entropy just to maintain the status quo; free energy must be supplied to sustain life.

Man has learned to utilize chemical energy in various kinds of heat engines to do work. In a steam engine the burning fuel supplies energy to do work and overcome losses to entropy. Yet, the process by which biological systems accomplish the same result, but at much greater efficiency and under much milder conditions, remains essentially unknown.

The living cell is not a heat engine but a chemical engine. Energy is stored as bond energy in the molecular components of cells and work is done or heat is released by breaking or rearranging these bonds. Energy transfer between two compounds must result either from direct interaction or from indirect interaction through a common intermediate. Phosphoric acid derivatives are related by common equilibria and are thus capable of trans-

fering chemical bond energy through common intermediates.

$$RNH_2^+-PO_3H_2 + R'OH = R'OPO_3H_2 + RNH_3^+$$

$$R'OPO_3H_2 + R''OH = R''OPO_3H_2 + R'OH$$

Phosphoric acid derivatives are of great importance, in this connection, throughout the phylogenetic scale, since they are intermediates in most metabolic reactions. Adenosine triphosphate, ATP, by virtue of its ability to phosphorylate metabolites, displaces equilibria of metabolic reactions with a net conservation of energy and in this way, supports all the energy requiring processes upon which life is based. ATP is the major "energy currency" of the cell.

The chemical energy contained in ATP maintains the integrity of the smallest living units and energy released by its hydrolysis or by phosphate transfer, is used to do osmotic, electrical and mechanical work (67). In addition, this energy is used in the synthesis of complex chemicals such as proteins, polysaccharides and the code of life itself, deoxyribonucleic acid. The ubiquitous ATP is found in every system classified as living and should be recognized as a vital ingredient in the reactions we call life.

Oxidative phosphorylation is the most important process by which the energy of foodstuffs is trapped in a biologically useful form, ATP. This process consists of metabolic oxidation-reduction via terminal

electron transport, with a concomitant synthesis of ATP from inorganic phosphate and ADP. It is a particularly efficient process because the energy liberated by the overall reaction is released in small increments during passage of electrons through a series of substances having graded chemical potentials. In this way, the energy of a high potential chemical reaction, such as the oxidation of glucose by oxygen, is distributed among a series of low potential reactions where efficient energy conservation may occur. The energy release in each step is small, the reactions are nearly reversible and maximum free energy production is approached.

The mechanism of oxidative phosphorylation is unknown. The process does not occur in the absence of highly ordered molecular systems, such as are found in intact mitochondria (58). Some mitochondrial fragments can perform oxidative phosphorylations but appear to possess the complete sequence of enzymes found in intact mitochondria (56, 50). Disruption of the order of this enzyme sequence causes the loss of oxidative phosphorylation. Hence, this reaction mechanism has been difficult to study and remains one of the major problems of biochemistry.

In model reactions, certain biologically active phosphate derivatives are converted to high transfer potential phosphates (37, 38). Circumstantial evidence suggests this function is retained *in vivo* (4). The purpose of this research is the application of modern physico-chemical techniques to a study of the mechanism of oxidative phosphorylation in model systems and in biological systems.

2. HISTORY

In 1907, Young (128) reported the formation of a hexose phosphate during fermentation of sugar by yeast. Subsequent discoveries of other phosphorylated sugars as fermentation intermediates (109) made it apparent that phosphorylated compounds were vital to biological function. The Eggletons showed a phosphorylated substance was hydrolysed during muscle contraction and reconstituted while the muscle was at rest (46). Meyerhof (95) observed that the hydrolysis of this phosphate, creatine phosphate, was accompanied by the release of much energy. Further work indicated that the energy released by hydrolysis was approximately equivalent to the work done by muscle (87). This was the first recognition that phosphate esters were capable of storing chemical energy.

Several high transfer potential phosphates were soon discovered, the most important of which was ATP. In studies of muscle contraction, Lohman (85) discovered that ATP hydrolysis preceeded the dephosphorylation of creatine phosphate. His conclusion that ATP was the substance directly responsible for energy storage became one of the major concepts of the modern biological sciences.

Engelhardt observed that two pathways for high transfer potential phosphate formation were present in most of the tissues and tissue extracts studied (47); an anaerobic process inhibited by fluoride and an aerobic process inhibited by cyanide but not fluoride. The full significance of these observations was first realized by Kalckar (72). He observed the phosphorylation of hexose in an oxygen-dependent system from kidney. Oxidation progressed in the absence of phosphorylation but not the converse. He later showed that addition of metabolic substances which increased oxygen consumption also increased the incorporation of orthophosphate into phosphate esters (73). His observation that the moles of phosphate ester formed exceeded the number of gram atoms of oxygen reduced was extremely important. If this occurred by a single process, it clearly distinguished oxidative phosphorylation from substrate level phosphorylations occurring during glycolysis.

The quantitative investigations of Belitzer and Tsibokawa (9) clearly defined oxidative phosphorylation. They showed that approximately two moles of orthophosphate were esterified in the high transfer potential form, phosphocreatine, for every gram atom of oxygen reduced. In addition, they pointed out that their tissue extracts also catalysed

hydrolysis of phosphate esters. Therefore, probably the P:O ratio (moles phosphate esterified: gram atoms oxygen reduced) was even greater than two. The energy released by the transfer of electrons from substrate to oxygen could theoretically produce from four to six phosphorylations, depending on the substrate employed. Belitzer proposed that oxidative phosphorylation was a mechanism for coupling the energy requiring steps of ATP synthesis to the energy releasing steps of electron transport.

Refinements in technique by Ochoa (102) made it possible to show that an average P:O ratio of three was maximum unless simultaneous substrate level phosphorylations occurred. Some uncertainty concerning maximum P:O ratios arose with the observation of Dallam that P:O ratios in excess of three could be obtained under certain conditions (43).

Lipmann (83) termed ATP an "energy rich" phosphate and described how this and other "energy rich" compounds, produced high free energies of hydrolysis. He defined "energy rich" compounds as those yielding more than 4 kcal/mole free energies of hydrolysis and laid the foundation for theories explaining why some phosphates yielded so much more free energy of hydrolysis than did others. Kalckar (74) also developed theories to explain the "energy

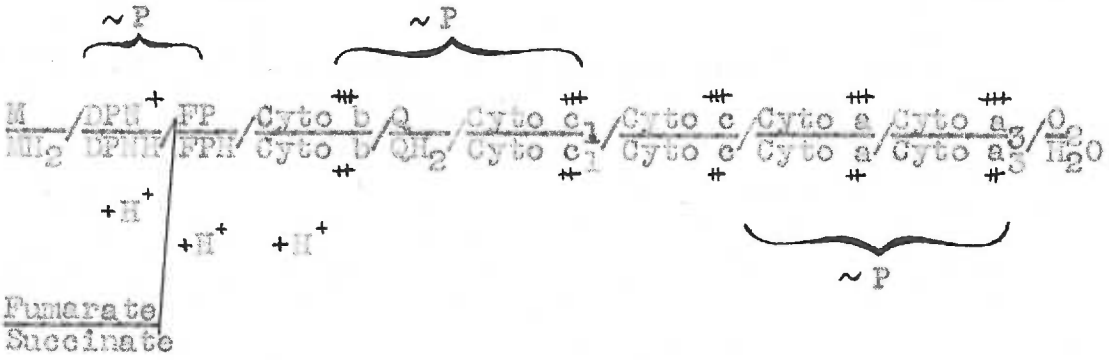
rich" character of these compounds. He explained that the large free energies of hydrolysis observed resulted from increased resonance among hydrolysis products and from the relief of static repulsion between components of the energy rich compounds. He further described how the transfer of phosphate groups from energy rich compounds to various metabolites resulted in biological synthesis. Oesper (105) later pointed out that the high free energy of hydrolysis was a function of pH. The free energy of hydrolysis increased with pH.

In 1948, Lehninger found the electron transport system and associated oxidative phosphorylation system were located in a particulate fraction, mitochondria, of homogenized cells (80). Mitochondria were complex, many functioned particles responsible for a major portion of cell catabolism. Only within the last few years has it become possible to obtain simpler systems still capable of synthesizing ATP (56, 50) and these mitochondrial fragments appeared to contain most of the enzymes originally present in the intact mitochondrion. Study of any particular reaction in such a complex system necessarily must be exceedingly difficult.

3. ELECTRON TRANSPORT

The electron transport system is composed of a complex group of enzymes located in mitochondria where its function is the transfer of electrons from various substrates to molecular oxygen. The purpose of this multiple transfer appears to be energy conservation, which results from oxidative phosphorylation intimately associated with the process (48). Numerous functions, such as ATP-dependent mitochondrial contraction (117), active accumulation of divalent cations (31) and active accumulation of phosphate (81), are performed by mitochondria in addition to oxidation-reduction and energy conservation. Consequently, it is difficult to relate experimental results to any single function.

While there is general agreement on the overall form of the electron transport system, a number of pathways and components are still in dispute (108). The diagram illustrates a formulation of the electron transport system. This sequence is based on oxidation potentials of the isolated enzymes and complex inhibition studies (30).



The redox potentials for several system components are based on two-electron oxidation-reduction reactions. Yet theoretical (97) and experimental results (82) indicate a one-electron transfer is more likely, particularly amongst the cytochromes. Since, in fact, biological electron transport entails one-electron transfer, other than that occurring between the cytochromes, recorded redox potentials may be incorrect. The E_h value for a two-electron oxidation is equal to the average of the two one-electron E_o values (39):

$$E_{o_t} = \frac{E_{o1} + E_{o2}}{2}$$

The E_{o1} for removal of the first electron may be very different from the E_{o_t} value observed in reactions involving two-electron transfers. One also must keep in mind that there is not complete agreement on either the sequence or components of the system.

Positions where oxidative phosphorylation presumably occurs, are shown in the electron transport scheme as outlined by West and Todd (124). These have been determined primarily by inhibiting or bypassing specific phosphorylation steps and observing oxidation states of the electron transport carriers (30). In the presence of substrate and oxygen, carriers preceeding the phosphorylation step are reduced and those following are oxidized. Calculations of available free energy from a reaction between the two carriers bracketting the oxidative phosphorylation indicate sufficient energy may be released to synthesize ATP. However, these free energy calculations are based on redox potential according to the familiar equation,

$$\Delta F = nf\Delta E.$$

Since ΔE is not known for many one-electron oxidations and since it may vary in the lipid mitochondrial medium from that observed in aqueous solution, derived ΔF values should be accepted with caution.

The coupling of energy release during biological oxidation, to synthesis of ATP remains a most mysterious process. In attempting to explain this phenomenon, the majority of investigators agree on two points. First, some member of the electron transport system is involved

directly in energy conservation. Second, the process of energy trapping is analogous to substrate-level phosphorylations and utilizes similar chemical compounds. A number of functional groups capable of participating in the energy conservation step have been discussed by Hunter (68). Among these are found quinones, which seem good candidates in view of recent developments (4, 37, 38, 61) which will be further developed below.

4. QUINONES IN ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATION

Quinones are widely distributed in nature, benzoquinone being found throughout the plant and animal kingdoms while naphthoquinones are found in some plants and many microorganisms (22, 41, 101, 23). As yet no direct evidence for naphthoquinone occurrance in mammals has been obtained but the work of Green (59) suggests its presence in beef liver. Organisms in which quinones have not yet been detected almost invariably are strict anaerobes (41).

Assay of quinone content has shown that the respiratory activity and concentration of quinone are directly related (41). Further, yeast grown anaerobically lose their quinone to an appreciable extent, regaining it when returned to an aerobic state. This strongly suggests an important respiratory function for quinone derivatives.

Localization of quinones within mitochondria of animals, chloroplasts of plants and particles analogous to mitochondria in microorganisms associates them exclusively with respiratory reactions.* Lowering naphthoquinone concentration by growing animals on a vitamin K deficient diet or by destroying quinone by U.V. irradiation, results in a lowering of oxidative phospho-

* A. F. Brodie, Personal Communication - 1963.

rylation efficiency.* Consequently, growth rate in microorganisms may be inhibited by light. Irradiated facultative anaerobes grow at characteristic rates on fermentable substrates but fail to grow on oxidizable substrate.

Many biological quinones have been detected and isolated (112). However, only a few benzo- and naphthoquinone derivatives have been implicated definitely in respiratory processes. Among these, there is a remarkable similarity of structure (41). Redox potentials are of the proper magnitude for their participation in electron transport at levels near the flavins (41). Thus their distribution, subcellular location and chemical properties imply a respiratory function for these quinones. Inhibition of respiration by their removal tends to confirm this view.

Naphthoquinone analogs possessing antivitamin K activity long have been known as potent respiratory inhibitors (5,120). In 1954, Martius showed that the cytochrome pigments, cytochromes b, c, and a, were rapidly reduced by naphthohydroquinone (88). In the presence of reduced nicotinamide dinucleotide (NADH) generating systems, rapid reduction of cytochromes c and

* A. F. Brodie, Personal Communication - 1963

a occurred in the absence of hydroquinone but cytochrome b reduction was very slow. In this case, cytochrome b reduction was stimulated by the addition of quinone and the stimulation counteracted by dicoumarol. It was shown that an enzyme in mitochondria mediated the transfer of electrons from NADH to vitamin K (89). This enzyme was extensively purified and was found to contain a flavin as prosthetic group. In addition, it was sensitive to several known inhibitors of electron transport and oxidative phosphoryation. Another enzyme isolated from dog liver had similar properties (126). However, both enzymes were found to be relatively non-specific, utilizing several dyes but only certain members of the vitamin K family. An enzyme, isolated by Hatefi, transferred electrons from NADH to coenzyme Q, a benzoquinone (64). Reports of this reduction *in vivo* also have been published (57).

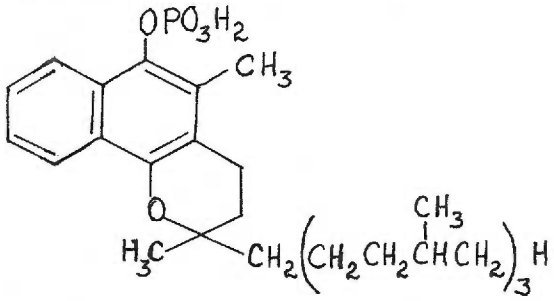
It was with respect to oxidative phosphorylation that the greatest interest centered on quinones. In the electron transport schome, positions where two of the three oxidative phosphorylations occurred were also the positions where quinones were implicated.

Martius observed that the efficiency of phosphorylation was lower in mitochondria from vitamin K deficient chicks than in those from control animals (90). This result could be reversed by the addition of vitamin K. Wessels outlined a mechanism by which vitamin K participated directly in oxidative phosphorylation while undergoing a cyclic oxidation-reduction. (123).

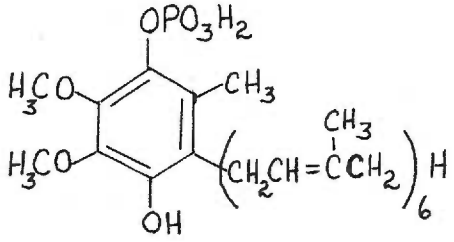
Continued interest has been shown in the effects of quinone depletion on oxidative phosphorylation and respiration. Considerable evidence has accumulated that relates oxidative phosphorylation, electron transport and quinones in plants and microorganisms.* In chickens or rats, the evidence is a great deal more circumstantial. However, it seems unlikely that a process as fundamental as oxidative phosphorylation would differ in major respects among plants and animals. One is reminded of the quote by Galileo, "Nature doth not that by many things which may be done by few."

* A. F. Brodie, Personal Communication. 1963.

Several investigators have observed the formation of high transfer potential phosphates or the transfer of phosphate groups during oxidation of synthetic quinol monophosphates (3, 4, 37, 38, 125) and have discussed the theoretical feasibility of such a reaction in biological oxidative phosphorylation. Recently Brodie observed the biological synthesis of ATP by M.phlei extracts and a phosphorylated derivative of vitamin K_1 (4) while Wieland observed ATP formation from the hydroquinone monophosphate of coenzyme Q in rat heart mitochondria (61), during oxidative phosphorylation.



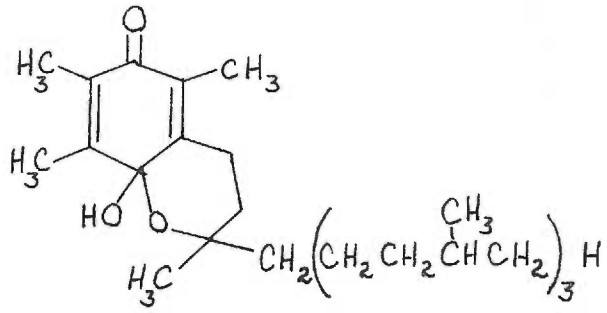
6-chromanyl phosphate of vitamin K_1



Hydroquinone monophosphate of Coenzyme Q

A serious disadvantage in considering quinones for an active part in oxidative phosphorylation is the failure to observe the required quinone in mitochondria and the difficulty in explaining how the quinols become spontaneously phosphorylated. However, it has been proposed that the active form of vitamin K in animals is

the cyclised chromanol form (33). Brodie has observed that the chromanol form of vitamin K is a requirement of oxidative phosphorylation in his biological system. Others (75, 65, 14) have shown that the chromanol form of benzoquinones is of possible importance. Boyer has shown that 6-chromanol derivatives may undergo two electron oxidations to form a metastable compound, tocopheroxide, which does not have the characteristic spectrum of a quinone (15). This substance may be reduced to the chromanol form again. Tocopheroxide was shown by Martius (91) to be a hemiacetal of tocopheryl quinone.

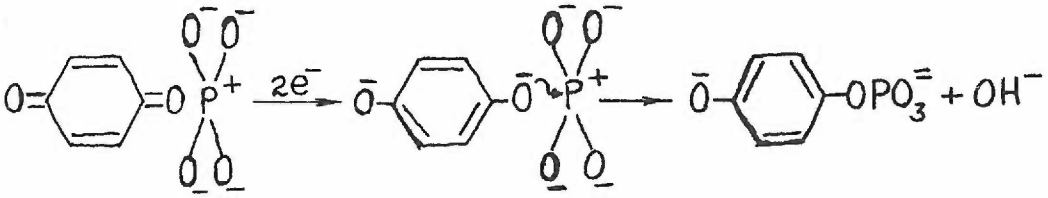


Tocopheroxide

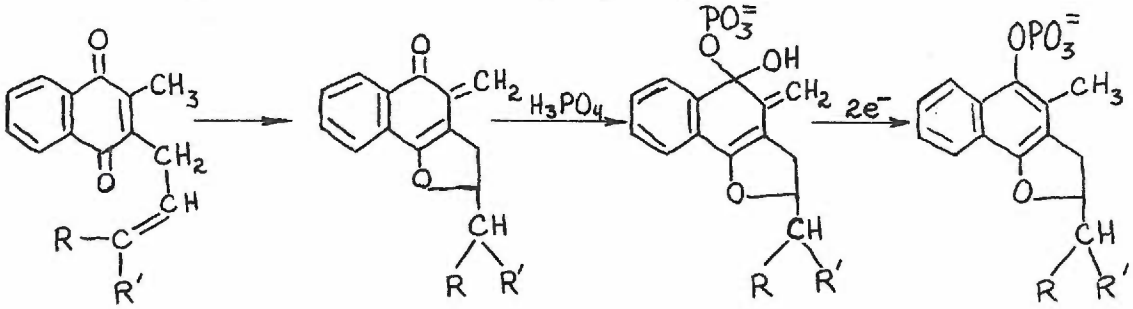
It is possible therefore, to have an active form of vitamin K within tissues which undergoes cyclic oxidation-reduction without producing a substance recognizable as a quinone.

The problem of the mechanism by which quinones may be phosphorylated to yield quinol phosphates has not been solved. Ingraham (69) proposed a reduction of quinone in

very close proximity to the phosphorus of a phosphate group. The hydroquinone anion formed a phosphate ester by nucleophilic substitution.

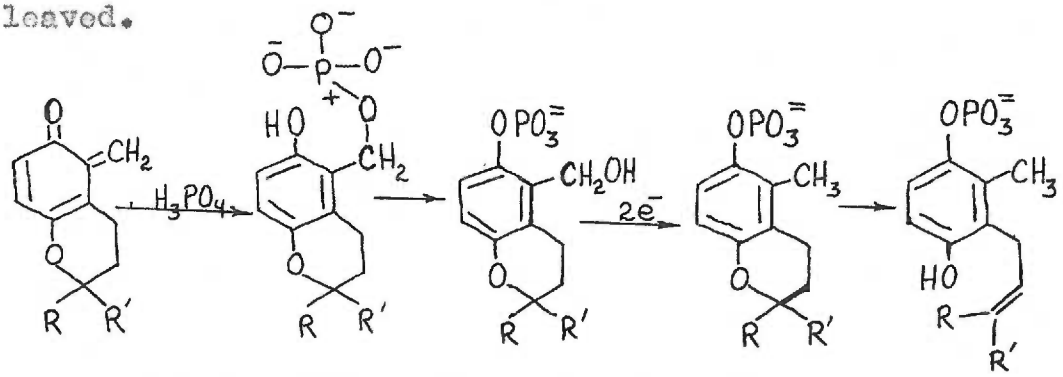


Chmielewska (32) proposed that a cyclization of the phytyl side chains of vitamin K during reduction led to a chromanol derivative. Phosphorylation followed by reduction produced a chromanyl phosphate.



Dallam (42) proposed a similar mechanism in which the chroman ring was opened by phosphorolysis after reduction. Lederer (118) proposed phosphorylation of the quinonoid methylene group followed by phosphate transfer to the oxygen. This was then reduced and the chroman ring

cleaved.



Other mechanisms for phosphorylating quinone derivatives have been proposed by Boyer (17), Cohen (40), Clark (36), and Brodie (24).

5. MECHANISMS OF OXIDATIVE PHOSPHORYLATION

Mitochondrial structure appears to play an important role in oxidative phosphorylation and attempts to simplify phosphorylating systems by removing unrelated enzymes have failed repeatedly (78). Consequently, experiments on biological oxidative phosphorylation are complex and difficult to interpret conclusively. Several mechanisms have been proposed which explain particular experimental results in terms of the more familiar substrate level phosphorylative reactions. While most of these general mechanisms are similar, they differ on very important points. This diversity in hypothetical mechanisms makes it obvious that very little is known about the actual mechanism of biological energy conservation. For this reason one cannot afford to overlook any experimental data nor artificially classify its importance.

A general mechanism for oxidative phosphorylation was first proposed by Lipman in 1946(84). This mechanism is analogous to the substrate level phosphorylating system utilizing phosphoenolpyruvate. A modern version of this process is outlined below.

$$AH_2 + X + Pi = A + XH_2 \sim P$$

$$XH_2 \sim P + B = X \sim P + BH_2$$

$$X \sim P + ADP = X + ATP$$

The symbols A, AH_2, B and BH_2 represent the oxidized

and reduced forms respectively of respiratory carriers. Orthophosphate is represented by P_i. Attempts to explain the results obtained with quinone derivatives must involve modified forms of this mechanism.

Another general mechanism was proposed by Slater in 1953 (110), analogous to the substrate level phosphorylation involving oxidation of glyceraldehyde -3- phosphate.

$$AH_2 + B + X = A \sim X + BH_2$$

$$A \sim X + P_i = A + X \sim P$$

$$X \sim P + ADP = X + ATP$$

Lehninger used this mechanism to explain results of some of his P^{32} exchange experiments (77). Variations of it are still favored and seem to have gained general acceptance.

Chance (29) showed that Lehninger's exchange experiments could be interpreted in still another fashion. He proposed a general mechanism based on results of inhibition studies in which energy conservation was a reductive process.

$$B + I = BI$$

$$BI + AH_2 = BH_2 \sim I + A$$

$$BH_2 \sim I + X = BH_2 + X \sim I$$

$$X \sim I + P_i = X \sim P + I$$

$$X \sim P + ADP = ATP + X$$

Energy conservation by a reductive step is not impossible.

However, analogous aerobic reactions have not been observed.

Boyer (16) suggested three generalized mechanisms for oxidative phosphorylation. He reasoned that ATP could be formed from ADP and orthophosphate (P_i) directly, from ADP by a nucleophilic attack on a phosphate ester or from orthophosphate by a nucleophilic attack on an ADP ester.

(1) $ADP + P_i = ATP + H_2O$

(2) $P_i + Y = P - Y$

$P - Y + ADP = ATP + Y' + H_2O$

(3) $ADP + Y = ADP - Y$

$ADP - Y + P_i = ATP + Y' + H_2O$

Mitchell (99) proposed a unique mechanism for oxidative phosphorylation. Oxidation of substrate was believed to cause an increase in intermitochondrial H^+ concentration.

$AH_2 = A + 2e^- + 2H^+$

Oxygen was reduced with the concomitant formation of OH^- outside the mitochondrial membrane.

$O_2 + 4e^- + 2H_2O = 4\ OH^-$

Combination of these ions was prevented by the semipermeable membrane and a large potential gradient developed. H^+ ions within the mitochondrion, combined with

OH^- produced through the formation of ATP and constituted the driving force of the reaction.

$$ADP^{-3} + HPO_4^{-2} = ATP^{-4} + OH^-$$

The two general mechanisms of Lipmann and Slater seem to best satisfy requirements of the natural oxidative phosphorylation process but there is yet insufficient evidence to allow an assignment of precedence between the two (107).

A number of mechanisms for oxidative phosphorylation were proposed in which specific compounds, known to be intimately associated with the natural process, were involved. In general, these were based on similarities of structure between electron transport substances and those involved in the analogous reactions of substrate level phosphorylation. For obvious reasons compounds having carbonyl groups were given special consideration.

George (51) proposed a mechanism, similar to that of Orgel (104), in which a group strongly bound to a heme-iron in the reduced state became weakly bound in the oxidized state and thus, easily transferable. The free energy of oxidation was conserved by the formation of this high transfer potential compound. A mechanism proposed by Eyring (115) also involves electron transport through complex heme compounds.

In 1959, Lindberg et al.(82) proposed a mechanism whereby phosphate was incorporated into reduced flavin during a reductive step. This was promoted to a high transfer potential phosphate by reoxidation. A more specific presentation of this mechanism was published by Grabe in 1960 (55). Glahn and Nielsen (53) explored the possibilities of forming high transfer potential compounds through oxidation-reduction of flavins, pyridine nucleotides and hemes. Recently a high transfer potential phosphate derivative of NAD was reported (60). Such a compound was proposed as an intermediate of oxidative phosphorylation by Lehninger in 1954 (76).

Lehninger (79) proposed another mechanism involving quinones but not requiring the participation of phosphate or phosphorylated compounds in electron transport.

(1) quinol-X = quinone ~X

(2) quinone ~X + E = quinone + E ~X

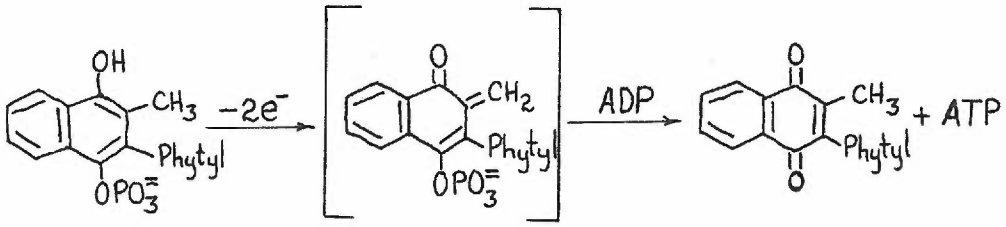
(3) E ~X + P_i = E ~P + X

(4) E ~P + ADP = ATP + E

Among speculative mechanisms proposed, those of Boyer (17), Cohen (40), Slater (111), Dallam (44) and Chmielowska (32) suggested direct participation of phosphorylated quinone derivatives in oxidative phosphorylation. Other mechanisms, similar to these and

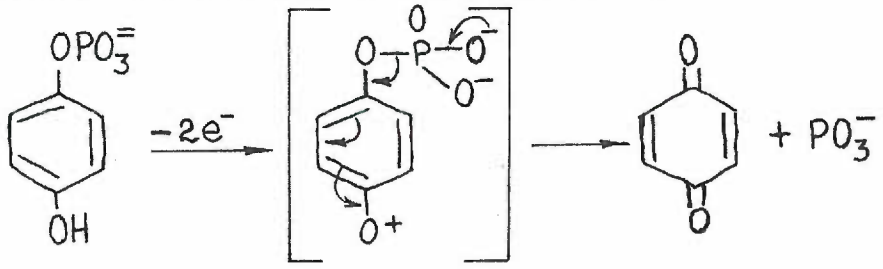
based on that of Clark, have been reported. The theme of these papers dealt primarily with phosphorylation of quinols and therefore, they have been previously discussed under that heading.

Wessels first proposed a mechanism for oxidative phosphorylation involving the oxidation of a hydroquinone phosphate (123).



A model oxidative phosphorylation system was reported by Clark et al (34, 37), who tested Wessels' mechanism. Phosphate derivatives of various reduced quinones were oxidized to yield the parent quinone and high transfer potential phosphates. Control experiments showed that no significant hydrolysis of these phosphates occurred in the absence of oxidation. Upon oxidation, as high as 50% of the phosphate could be recovered in high energy forms accompanied by high yields of the quinone. Adenosine diphosphate (ADP) was synthesised from adenosine monophosphate (AMP) by this method in a later experiment (35, 38). A mechanism for the

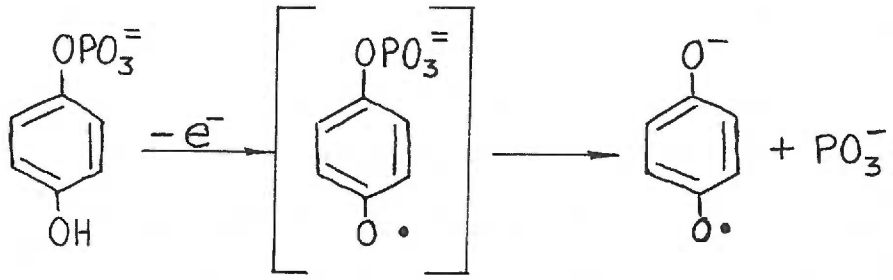
reaction was proposed (37) similar in some respects to that suggested earlier by Wessels (123).



The primary phosphorylating agent was believed to be the hypothetical monomeric metaphosphate (PO_3^-). This substance has never been detected in a chemical reaction although Westheimer (25) Jencks (71) and Todd (115) support its presence as an intermediate on kinetic grounds.

Wieland and Pattermann (125) performed experiments similar to those of Clark. They oxidized a quinol phosphate with iodine in ethanol and reported the formation of ethyl phosphate. It was concluded that a high transfer potential phosphate compound was formed as an intermediate. This experiment showed a high efficiency transfer of phosphate from quinol to ethanol during oxidation but did not show the formation of a high transfer potential phosphate. The observed transfer is energetically favorable even in the absence of oxidation. They proposed a mechanism for the reaction similar to that of Clark.

The formation of a high energy intermediate in these reactions is usually explained in terms of a two-electron oxidation mechanism. In 1958, Harrison published arguments in favor of a one-electron mechanism leading to the formation of a high energy intermediate (63).



It was argued that the intermediate, semiquinone phosphate, would have a high transfer potential by virtue of the increased resonance gained upon dissociation into a symmetrical semiquinone anion.

Mason observed that the benzo-1,4- semiquinone anion was not protonated at pH 4.8 (127) while Yamazaki* observed that its five line ESR spectrum changed near pH 3 as a result of protonation. Yamazaki concluded that this semiquinone was approximately as strong an acid as acetic acid. By analogy, semiquinone phosphate should have a free energy of hydrolysis comparable to that of acetyl-phosphate. This evidence confirmed Harrison's postulate concerning the high energy properties of a semiquinone phosphate. Mason** observed the oxidation of quinol

* I. Yamazaki, Personal Communication. 1963
** N. S. Mason, Personal Communication. 1963

phosphates by hydrogen peroxide and horse-radish peroxidase, a system known to catalyze only one-electron oxidations (28). Some of the oxidizing agents employed by Clark's group (37), such as ceric sulfate, also preclude all except one-electron oxidations and a high transfer potential semiquinone phosphate is a probable reaction intermediate.

Biological oxidative phosphorylation and electron transport through the cytochrome system are intimately linked. Cytochromes alternate between the ferrous and ferric oxidation states of the iron while flavins associated with electron transport form flavin semiquinones (8). Both processes require univalent changes. It is such evidence that makes the formation of radical intermediates during oxidative phosphorylation highly credible.

One objection to these proposed mechanisms is that they were not supported by experiment in most instances. Those for which experimental evidence was gathered do not necessarily describe biological systems since only simple in vitro reactions were studied.

In 1962 Brodie (4) studied the effects of Vitamin K_1 on oxidative phosphorylation in extracts of M.phlei. In this system, it appeared vitamin K_1 was reduced, cyclized to a chromanol derivative, phosphorylated and the phosphate group transferred to ADP during oxidation. This

was the first conclusive proof that quinone derivatives were functional in biological oxidative phosphorylation. His proposed mechanism was based on that of Clark.

Wieland (61) recently reported that rat heart mitochondria oxidized the hydroquinone monophosphate of coenzyme $Q_{(6)}$ with the concomitant formation of ATP. P^{32} labeled hydroquinone phosphate transferred the phosphate group to ADP in this reaction without dilution by added inorganic phosphate. Of three related hydroquinone monophosphates tested, only the coenzyme Q derivative was active with these mitochondria.

Recently, a number of high transfer potential compounds capable of converting ADP and orthophosphate to ATP in vivo, were reported (60, 18, 119, 106). There was little doubt that these compounds coupled energy conservation to the synthesis of ATP but whether they functioned as primary or secondary coupling agents could not be determined.

6. PRODUCTION AND DETECTION OF FREE RADICALS

A number of techniques have been devised for trapping short-lived radical intermediates in organic reactions (6). Such radical intermediates may be detected or identified, without disturbing the reaction system, by electron spin resonance spectroscopy (70). By this technique, Harrisons' hypothesis, that semiquinone phosphate is an intermediate in the model reaction, should be tested easily. This semiquinone phosphate may also be sought in actively phosphorylating mitochondria. The appearance of this radical in mitochondria would be very good evidence that the model reaction represented the biological reaction.

Substances known as free radicals are not found in appreciable concentrations except under unusual conditions. They are usually very reactive, combining readily with other radical species to form more stable non-radical compounds. A free radical has an unpaired electron. From a stable molecule having only paired electrons, a radical may be produced by adding an electron as in reduction or by removing an electron as in oxidation. Reductive synthesis is the method of choice because of controlled, essentially quantitative conversion (94, 2).

Oxidation reactions are less easily controlled and

frequently some degree of decomposition occurs. However, some experiments require oxidation and in these instances, it is necessary to seek conditions producing the least decomposition.

One chemical substance will oxidize another having a lower reduction potential but the rate of reaction may be negligible. Because radicals are reactive and have short half-lives, it is necessary to have a high generation rate for detectable quantities to be obtained. This rate may vary with ionic strength, temperature, complexing agents or pH (51). The principle of equivalent change also plays an important role in the rates of some chemical reactions. Slow rates due to incompatibility of oxidant and reductant may usually be overcome by choosing a different oxidant or by employing a mediator.

Mediating substances, otherwise known as catalysts, react readily with both oxidant and reductant. The activation energy for a particular reaction is lowered so that the reaction rate is increased and reaction character is modified. Such a catalyst is the enzyme, horse-radish peroxidase. It mediates the reaction between hydroquinone and hydrogen peroxide yielding almost quantitative amounts of quinone by a fast reaction.

Without this catalyst, the reaction rate is slow and the product is a mixture of epoxyquinones, quinone and polymer. An important property of peroxidase is its ability to oxidize substrates by single-electron transfer (28) to yield free radicals exclusively.

An inert electrode of the proper potential may serve as an oxidizing or reducing agent as effectively as chemical reagents. Electrolytic reductions in both aqueous and nonaqueous solution have been studied (2). These reductions are easily controlled and usually lead to quantitative recovery of product.

On the other hand, electrolytic oxidations have been studied but results have been inconsistent. As yet, they are treated empirically and the mode of action, like that for most of the chemical oxidizing agents, is not understood.

Reactive radicals produced by any of the means described must be trapped in a non-reactive form if they are to be studied. Most commonly, the reaction solution is frozen, trapping radicals in an ice matrix where they may not interact (19, 6). In other kinds of solution, the radicals may be trapped in a glass or plastic. In addition, some radicals may be converted to a stable form by manipulation of pH (96).

Free radicals are paramagnetic species and these react

to an applied magnetic field by being attracted to it. Magnetic susceptibility measurements determine the number of unpaired electrons; consequently the concentration of radicals may be determined.

Michaelis (96) detected radical species by observing the redox potentials as a function of percentage oxidation and by observing their intense optical absorption spectra. Both methods are more sensitive than magnetic susceptibility measurements but neither will identify a radical species. Electron spin resonance spectroscopy (ESR) possesses the sensitivity of optical spectroscopy and the capacity to make a positive identification of radical species. Techniques have been improved for handling highly reactive radicals and for detecting extremely low concentrations of them (116) making ESR a superior instrument for studying radical reactions.

7. PRINCIPLES OF ELECTRON SPIN RESONANCE

Electrons possess both electric charge and an intrinsic angular momentum called spin. A magnetic moment, μ, is associated with the electron and is related to the angular momentum and electric charge by the expression,

$$\mu = g \frac{eh}{4\pi mc}$$

C is the velocity of light; h, Plank's constant; e, the electric charge; m, the mass of the electron and g, a constant measuring the contribution of spin and orbital motion to the angular momentum. The quantity, $\frac{eh}{4\pi mc}$, usually denoted by β , is a constant called the Bohr magneton (70).

An applied magnetic field, H, may orient μ by interacting with it. Only two orientations are allowed, however, characterized by the spin quantum numbers, $M_s = \pm \frac{1}{2}$. The energy of interaction between μ and H is given by the expression, $E = M_s \mu H$. This is more commonly written, $E = M_s g \beta H$.

In the absence of an applied magnetic field, both spin states, corresponding to the two orientations in a field, are of equal energy; that is, they are doubly degenerate in spin (26). When placed in a magnetic field, the two spin states lose their degeneracy. Electrons whose magnetic moments oppose the applied field, are said

to be antiparallel oriented and have higher energy than those whose magnetic moments complement the field. These latter are said to be oriented parallel to the field.

The difference between two energy levels corresponding to the two orientations in a magnetic field is a function of the applied field. This is illustrated in Fig. I.

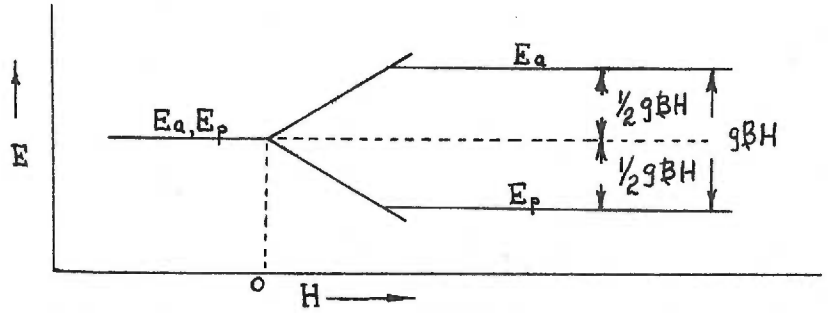


Fig. I

E_a is the energy of the antiparallel spin state and E_p, that of the parallel spin state. It is seen that the difference in energy between the two spin states is, $\Delta E = g \beta H$. For a free electron, the spectroscopic splitting factor, g, is equal to 2.0023. Electrons in the parallel spin state may absorb electromagnetic radiation of the proper energy and be raised to the higher energy state. Since the energy of a photon is described by the expression, $E = h\nu$, where ν is the frequency of electromagnetic radiation, an expression describing condi-

tions necessary for a transition between electron spin states is, $h\nu = g\beta H$. From this equation, it is seen that two variables, H and ν , determine the conditions necessary for electron spin resonance.

An electron spin resonance spectrometer measures energy absorption when electrons in the lower energy state are excited to the higher state. Resonance conditions are brought about usually by placing the unpaired electrons in a powerful magnetic field provided by a variable strength electromagnet and then directing a source of electromagnetic radiation toward them. This radiation source is usually a constant frequency klystron oscillating at 9 Kmc. Under these conditions, resonance occurs at about 3 Kgauss for a free electron or a free radical. The recorded signals are usually derivatives of the actual absorption curve unless a special integrating recorder is used. Therefore, it is necessary to integrate the signal graphically in order to observe the absorption curve. Radical concentrations are determined by comparing the areas under the absorption curves for the unknown and a standard solution of radicals.

Electrons in most stable atoms or molecules are paired, their spins cancel each other and the substances

are diamagnetic. In accordance with the Pauli exclusion principle, no changes in spin state may occur under any conditions unless the electron is promoted to a new orbital. Such conversions do occur but this triplet state need not be discussed here. Some substances such as free radicals, heavy metal ions and certain stable molecules are paramagnetic; that is, they contain unpaired electrons. These substances can be made to undertake changes in the spin states of their electrons and are subject to study by electron spin resonance spectroscopy.

Conditions which stimulate the absorption of energy and a change of spin to a more energetic state also stimulate the reverse. In order to detect a net absorption of power, it is necessary to have a greater number of electrons in the lower energy state than in the higher. At equilibrium, an unpaired electron will follow a Maxwell-Boltzmann distribution between its two spin states (70). If N_1, represents the higher energy state and N_2, the lower, their ratio is described by the equation,

$$\frac{N_1}{N_2} = e^{-\Delta E/kT}$$

ΔE represents the difference between transition energy levels, k is the Boltzmann constant and

T, the absolute temperature. Therefore, the lower the absolute temperature, the smaller is the ratio $N_1:N_2$ and the greater will be the power absorption under resonance conditions.

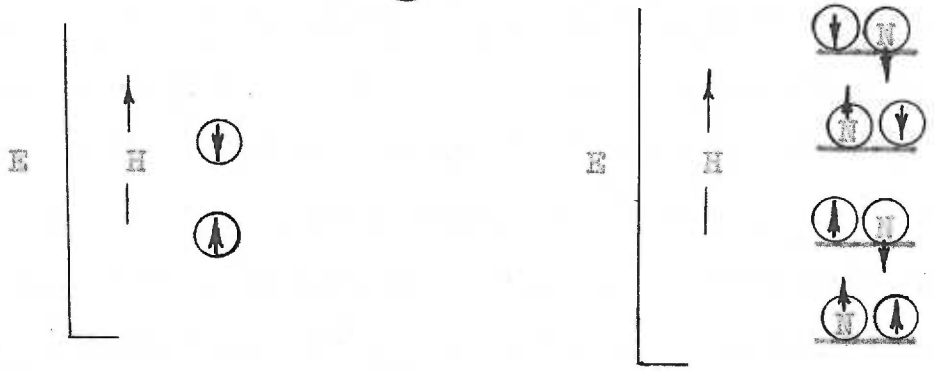
8. FREE RADICALS STUDIED BY ELECTRON SPIN RESONANCE

In the previous chapter, it was shown that electron spin resonance will occur at a definite magnetic field strength for any given value of "g" since the microwave frequency remains constant. The "g" value may change for different paramagnetic substances due to differing orbital contributions to the angular momentum. In addition, the apparent "g" value may vary due to electric field orientations, as in some transition metal ions. For this reason, many paramagnetic species may be differentiated by their apparent "g" values. However, for most free radicals, "g" does not vary widely from the value for a free electron (70). Thus a "g" value very near 2.0023 is indicative of the free radical family but will not distinguish one radical from another.

Identification of free radicals may be made through variations in their absorption spectra called the hyperfine spectra (70). This results from interactions between the magnetic moment of the unpaired electron and the magnetic moment of some nuclei. Interactions of this kind lead to a splitting of parallel and antiparallel energy levels into groups of sublevels at approximately the same energy. Instead of one transition energy,

there may be several; consequently several absorptions occur at slightly different values of the magnetic field, H. Hyperfine spectra represent interactions characteristic of a given molecule and therefore are characteristic of a given radical.

In order to obtain hyperfine spectra, the electron must interact with other magnetic materials. Fortunately, many of the atoms usually found in organic free radicals have nuclear magnetic moments. The most important of these are hydrogen and nitrogen nuclei but the phosphorus nucleus must not be overlooked. These nuclei line up parallel or antiparallel to an applied field just as do electrons. Possible arrangements of an electron's magnetic moment, ⊕, with respect to the applied field are shown in Fig. IIa. In IIb, the additional interaction with a magnetic nucleus, (N), is considered.



a. Fig. II b.

The only allowed transitions are between levels having the same nuclear spin. For a nuclear spin, M_n, it is seen that electronic transitions are allowed only when $\Delta M_n = 0$. Nuclear spin change does not occur during electron resonance, therefore only two transitions may occur in Fig. IIb even though four energy levels exist.

An electron will be influenced by every nucleus encompassed by its molecular orbital and will interact with each magnetic moment of these nuclei. These interactions will be characteristic of the molecular structure and the influence on available energy levels will be manifested by a characteristic hyperfine spectrum. Fig. III, illustrating the interaction between one unpaired electron and two nuclei having magnetic moments, describes the energy levels and possible transitions for the hydrogen molecule ion, H_2^+ (26).

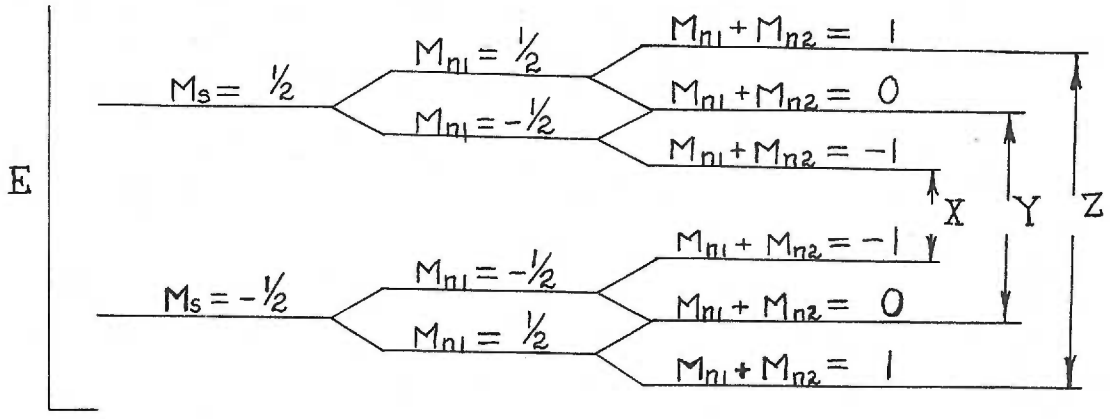


Fig. III

In Fig. III, the notation, M_{n1}, represents the spin quantum number for nucleus one and M_{n2}, the spin of nucleus two. Each of these may be oriented parallel or antiparallel to the field. It can be seen that two possible arrangements lead to $(M_{n1} + M_{n2} = 0)$ for the H_2^+ ion. These two levels have equal energy and are degenerate. Their populations are approximately double that for the non-degenerate levels and the probability of transitions occurring at any time is twice as great. Fig. IV is an example of the ESR absorption spectrum from H_2^+.

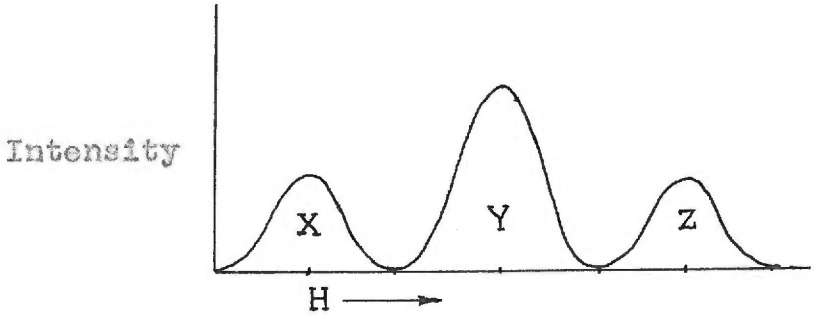


Fig. IV

X, Y, and Z represent the respective transitions in Fig. III. If an extension of these principles is made, it is found that the number of transitions, hence hyperfine lines, may be predicted from the equation,

Hyperfine lines = $2n(s) + 1$, where n is the number of nuclei interacting and (S) is their nuclear spin number. The intensities of the absorptions will be in the ratio of coefficients of a binomial expansion.

Two factors are responsible for the interaction between an unpaired electron and a nuclear magnetic moment. These may be described by an isotropic and an anisotropic term (70). The anisotropic term describes the classical interaction between the two magnetic dipoles. This interaction takes on a distinct value only in a single pure crystal. Even in this instance, the amount of interaction varies with the crystal orientation in the applied magnetic field. If the paramagnetic species is randomly distributed as in powders, viscous solvents or in ice, the anisotropic interaction is continuous and a broad ill-defined spectrum results. Most hyperfine structure is lost. Since hyperfine structure is crucial in identification of radical species, study of radicals trapped in glasses or ice cannot lead to a positive identification. Increased ease in detection and handling of materials does make low temperature trapping of radicals in ice an important quantitative analytical tool (19).

The isotropic term is derived from relativity theory. It is based on a Dirac delta function describing the three dimensional normalized distance between an electron and a magnetic nucleus. This term will only have a non-zero value if there is a finite probability of finding the electron at the nucleus. Only s- orbitals or some hybrids

will have a probability of finding the electron at a nucleus. Hyperfine splitting will occur if the molecular orbital has some s- character on the nucleus in question. Other types of orbitals have a zero node at the nucleus and no hyperfine interaction will occur.

Hyperfine interactions are observed from a number of substances where s- character in the molecular orbital encompassing magnetic nuclei would not be expected. This has been explained as due to configurational interaction (70). This results from a small degree of mixing between a ground state having no s- character and an excited state where some s- character is evident. The electron then assumes a finite probability of being at the nucleus and hyperfine interaction occurs.

Configurational interaction is extremely important in the study of aromatic substances where an electron travels in an orbital having a zero node in the plane of the magnetic nuclei. Because of configurational interaction, a degree of unpaired electron density finds its way into the σ bonds of the carbon nuclei. There it interacts with magnetic nuclei connected to the carbon by σ bonds. The configurational interaction leads to a transmission of unpaired electron density from a π molecular orbital through the σ orbitals to the mag-

netic nucleus where it interacts. The resulting isotropic hyperfine interaction is clearly defined and characteristic of the radical studied.

In non-viscous solutions, the molecules tumble rapidly with respect to the microwave radiation frequency and anisotropic interactions cancel. Only the sharp clearly defined isotropic hyperfine structure remains. This is highly characteristic of the radical and can be used for a positive identification.

9. INTERPRETATION OF ESR SPECTRA

Two factors determine the magnitude of interaction between protons on an aromatic system and an unpaired electron in the system. These are the unpaired electron density on the aromatic carbon adjacent to the proton and the coupling constant which is a measure of configurational interaction (70). If one assumes the coupling constant does not change for any proton in the system, the equation for hyperfine splitting may be written:

$\Delta H = Q\rho$. ΔH is the magnitude of the hyperfine splitting in gauss, Q is a constant for C-H units in aromatic systems and ρ is the unpaired electron density on a carbon adjacent to the proton responsible for hyperfine interaction. Calculation of unpaired electron density on aromatic carbons is a difficult process and, in many instances, insufficient data is available for an accurate calculation to be made. The splittings observed in the hyperfine spectrum of an aromatic radical therefore may be difficult to confirm by calculation alone. Conversely the densities may be determined easily from the observed splittings thus providing valuable data on electron distribution in aromatic systems. A recent review by Carrington (27) considers quantitative calculations.

For a semiquinone anion, the electronic structures may be represented as in Fig. V, where the asterisk represents the unpaired electron:

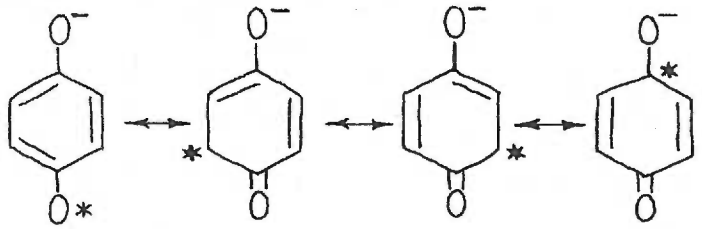


Fig. V

Since the molecule is completely symmetrical, there are four other combinations and it would appear that the unpaired electron is distributed over the entire molecule in such a way as to make each of the proton positions equivalent.

If one of the oxygens is prevented from conjugating with the system, the resulting molecule will behave as a phenoxy derivative with some perturbation from the inhibited oxygen.

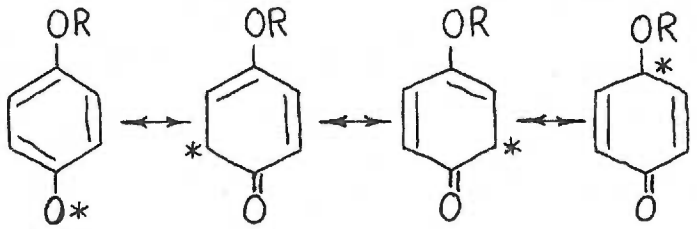


Fig. VI

It may readily be seen that the greatest unpaired spin

density occurs on alternate hydrocarbons. From this treatment, it would appear that the carbons without asterisks would have no unpaired spin density. However, hyperfine interactions at these carbons do occur although they are of smaller proportions than the others. They have been explained in terms of negative spin densities (27).

Functional groups other than protons also respond to an unpaired electron but the coupling constants are different. Methyl or methylene protons produce hyperfine structure in the spectrum of an aromatic radical though the protons are not in direct contact with an aromatic carbon. This interaction is due to hyperconjugation (70) and is approximately equivalent to the proton configurational interaction.

II EXPERIMENTAL

1. MATERIALS AND EQUIPMENT

A. Chemicals

Water for all experiments was condensed from steam lines and passed through a Barnstead standard mixed-bed ion exchange column to remove heavy metal salts.

Buffers were prepared as described in Methods of Enzymology, volume I (54), except for pH 11.7 phosphate buffer. This was prepared by mixing equal volumes of 0.2 M di- and trisodium phosphate followed by a 1:1 dilution with distilled water. All buffers were checked against Coleman pH standards.

A stock solution of 0.1 M H_2O_2 was prepared by diluting Baker reagent grade 30% H_2O_2 with distilled water. This was standardized against a standard solution of $KMnO_4$ by the usual method (114). Experimental solutions were prepared by dilution of this stock solution.

A stock solution of 0.2 M $KMnO_4$ was prepared from reagent grade $KMnO_4$, standardized and stored by the usual procedure (114). Experimental solutions were prepared by diluting with buffer of the proper pH.

A stock solution of ceric sulfate was prepared by

dissolving ceric ammonium sulfate in concentrated H_2SO_4 and diluting with distilled water to make a solution 0.1 M in ceric sulfate and 1.0 M in sulfuric acid. Further dilutions were required in preparation of experimental solutions. Chlorine solutions were prepared by acidifying "Chlorox" hypochlorite solution and diluting with 0.01 M H_2SO_4. Hypobromite solutions were prepared by dissolving the appropriate quantity of liquid bromine in an alkaline buffer or 0.1 N NaOH . Chloranil solutions were prepared by dissolving Eastman chloranil in absolute ethanol and diluting with the appropriate quantity of distilled water or buffer.

Quinhydrone, melting point 170.5--171°C, was dissolved in 0.1 M phosphate buffer, pH 7.2, to make a 5mM solution. This contained 6.5×10^{-6} M free radical (93) and was used as a standard for the determination of other free radical concentrations.

Chemicals generously supplied by Roche Products Ltd. through the kindness of Professor Lord Todd, Cambridge University, and used without further purification were, 4-hydroxy-2, 3-dimethylnaphthyl-1-dihydrogen phosphate; 4-hydroxy-2-methylnaphthyl-1-hydrogen phosphate, triethyl ammonium salt; 4-hydroxynaphthyl-1-hydrogen phosphate, cyclohexylammonium salt; 4-hydroxy-2,3,5,6-tetrachloro-

phenyl-1-dihydrogen phosphate, and 4-hydroxyphenyl-1-dihydrogen phosphate.

Roche Products also kindly supplied 4-hydroxy-3-chloro-2-methylnaphthyl-1-dibenzyl phosphate and 4-hydroxy-2,3,5,6-tetramethylphenyl-1-dibenzyl phosphate which were hydrogenolysed by the method of Clark (37) to produce the free acid. 4-Hydroxy-3-chloro-2-methylnaphthyl-1-dihydrogen phosphate melted with decomposition at 143 - 145°C. This slightly pink, crystalline solid appeared to darken when exposed to light or air in an open vessel. The other ester, 4-hydroxy-2,3,5,6-tetramethylphenyl-1-dihydrogen phosphate, was a white crystalline material. It appeared stable in air but exposure to light was avoided. Its melting point was found to be 235 - 237°C. with decomposition.

Turnip peroxidase was provided as a 7.8×10^{-5} M solution with an R.Z. of 2.0 through the generosity of Dr. I. Yamazaki, Tohoku University. Dr. C. A. Dekker, of the Department of Biochemistry, University of California, Berkeley, graciously provided 2-hydroxyphenyl-1-dihydrogen phosphate which was used without further purification. A system capable of performing oxidative phosphorylations, consisting of Mycobacterium phlei particles, supernatant and the 6-chromanyl phosphate of vitamin K_1,

was kindly provided by Dr. A. F. Brodie, Department of Medical Microbiology, University of Southern California Medical School.

α-Tocopheryl phosphate, disodium salt, was purchased from Calbiochem. Triethylaminoethylcellulose (TEAE- cellulose) was purchased from Bio-rad Laboratories.

B. Equipment

Below a value of 10, the pH of buffers was determined by a Beckman zeromatic pH meter equipped with a standard glass electrode. For higher pH values, a Beckman GS portable pH meter with a type E2 blue glass electrode was used.

All visible and ultraviolet spectrophotometric analyses were performed with a model 14 Cary recording spectrophotometer equipped with a water-jacketted cell compartment and cell holder to maintain constant temperature. A syringe drive and lucite mixer, described in Fig. VII, was used with a Varian quartz ESR cell, positioned with a special holder, for flow experiments.

A special apparatus (Fig. VIII) designed for spectrophotometric study of rapid enzymatic reactions, was used to add small amounts of enzyme to a mixture of substrate and hydrogen peroxide. Study of the reaction in very

early stages is possible with this apparatus.

Electron spin resonance spectroscopy was performed with a Varian model V4500 EPR spectrometer with 100 kc field modulation. This was used with a Varian liquid nitrogen accessory, a Hewlett Packard klystron frequency meter and a Varian F8 nuclear fluxmeter coupled to a Hewlett Packard 524 C electronic frequency counter. The frequency counter gave an accurate measure of proton resonance for a precision determination of magnetic field.

Electrolysis was carried out in a Varian model V4556 electrolytic cell assembly with calomel reference cell attached (Fig. IXd). A controlled voltage DC power supply provided the electrolytic current. This electrolytic cell assembly was positioned so that the platinum electrode was centered in the ESR cavity. The platinum electrode was connected to the anode of the DC power supply.

Five types of quartz ESR tubes were used for studying radicals. These are depicted in Fig. IX. Solutions could be studied under any desired condition by employing these cells.

Free radical signals were recorded by the Varian G-10 graphic recorder when low temperature measurements were made. At room temperature, a Texas Instrument Co. Servoriter integrating recorder and Sanborn 320 dual

channel recorder were used at maximum sensitivities. Because of difficulties with the integrating recorder, reported spectra have been integrated graphically.

A rapid-flow rapid-freeze apparatus patterned on a similar device, described by Bray (19) and modified in our laboratory, was used to study radicals trapped in ice shortly after reaction initiation. This device and its operation are described in Fig. X.

Acquisition of a room temperature ESR spectrum of short-lived free radicals was made possible by a rapid-mix rapid-flow device. The syringe drive was the same used in spectrophotometric analysis but a different mixer was attached to the flow cell. This mixer is shown attached to the ESR flow cell in Fig. IXB. For handling large volumes of solution, a pneumatic flow device (Fig. XI) was substituted for the syringe drive. Stopped flow for observation of decay rates was made possible by a set of Valcor Engineering Corp. solenoid valves. These small volume solenoids were constructed so that no metal parts contact solutions passing through the valve.

Chromatography was performed in large glass battery jars at room temperature. No special precautions were taken to maintain constant temperature conditions.

Mitochondrial oxygen consumption was followed on a Gilson Medical Electronics Co. oxygraph with an uncovered 36 guage platinum electrode sealed in glass as described by Nagihara (62).

2. PROCEDURE

Two types of experiments were designed for the detection of free radical species formed by the oxidation of quinol phosphates. Electron spin resonance spectroscopy was employed for direct detection and identification of radical intermediates while optical spectroscopy was used for the detection of intermediates which need not be necessarily radicals. Either the appearance of new absorption maxima or significant differences in the rates at which quinol phosphates disappeared and quinone product appeared, were sought in the spectrophotometric experiments as indication of intermediate formation. For such analysis, absorption maxima and extinction coefficients must be known under experimental conditions.

The spectrum of a 10^{-4} M quinol monophosphate solution in 0.1 M buffer was determined for the range, 230-360 mμ. A spectrum for the corresponding quinone was found by dissolving an authentic sample of quinone in alcohol and diluting with buffer to make a 10^{-4} M solution. Several quinol monophosphates and their corresponding quinones were thus compared at several pH values.

Conversion of quinol monophosphate to quinone by peroxidase and hydrogen peroxide was observed in the

spectrophotometer by both static and flow techniques. The concentration of reagents at the instant of mixing for static experiments was 10^{-4} M quinol phosphate, 10^{-2} M hydrogen peroxide and 5×10^{-7} M turnip peroxidase. Quinol phosphate was mixed with hydrogen peroxide in a cuvette, placed in the spectrometer and a recording of absorption at the maximum was begun. Peroxidase, previously applied to the tip of a plunger device (Fig. VIII), was rapidly added and mixed. Oxidation was followed by observing diminishing absorption at the substrate maximum. Later experiments utilized 5×10^{-4} M concentrations of H_2O_2. Reaction rate was observed in the presence of 10^{-3} M $MgCl_2$ to determine the effect of divalent cations.

Flow experiments utilized 10^{-2} M quinol phosphate, 10^{-2} M H_2O_2 and 10^{-6} M turnip peroxidase. As in static experiments, quinol phosphate and H_2O_2 were mixed, while peroxidase was diluted with buffer and placed in a separate syringe. These solutions were mixed in a lucite mixer just prior to entering the cuvette. From the flow rate, the time after mixing at which the spectrum was determined, could be calculated.

Identification of products from these reactions was made by extracting the exhaust with hexane and analysing the U.V. spectrum. The hexane was removed in a vacuum, the

residue was recrystallized from ethanol and its melting point was determined. An I.R. spectrum was obtained from this product and compared with that from an authentic sample of the expected product. A quantitative estimate of yield was obtained from the optical density at a maximum.

Phosphate containing compounds were separated by paper chromatography by the method of Clark (37) and were detected with a color reagent by a technique outlined in Paper Chromatography and Paper Electrophoresis, (11). Reference spots of phosphate and pyrophosphate were used to identify phosphorylated products.

Experiments, similar to those performed in the optical spectrometer, were conducted in which reaction intermediates were examined by electron spin resonance. A mixture of quinol phosphate, H_2O_2 and peroxidase was prepared as for a spectrophotometric experiment, placed in an ESR tube and rapidly frozen. Examination by ESR for evidence of a free radical was made. This same mixture was produced in the rapid-mix rapid-freeze device by forcing quinol phosphate plus hydrogen peroxide from one syringe through a small mixer into which peroxidase was being forced from another syringe. The outflow of the mixer was ejected into precooled isopentane

and rapidly frozen at liquid nitrogen temperature.

Quinol phosphate in pH 4.5 buffer was placed on a TEAE-cellulose column prepared in an ESR room-temperature cell. A mixture of 10^{-4} M H_2O_2 and 10^{-6} M peroxidase was passed through this column positioned in the ESR microwave cavity. The magnetic field was varied in the region of free radical absorption while oxidants were passed through the column. A similar experiment was performed whereby a 0.01 M solution of chloranil was used as a mobile oxidant for 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate adsorbed on a TEAE-cellulose column.

Rapid-mix rapid-freeze experiments were repeated, using 10^{-2} M quinol phosphates and 10^{-2} M solutions of such oxidants as bromine, chlorine, hypobromite, chloranil, acid ceric sulfate or alkaline permanganate. For example, $KMnO_4$ was prepared in pH 11.7 phosphate buffer as was the quinol phosphate. These solutions were mixed and injected into cold isopentane at variable times. A wide variety of quinol phosphates were treated in this way and their frozen solutions examined at low temperature for evidence of free radicals. A series of experiments were repeated in which quinol phosphate was prepared in 0.01M ceric sulfate in 0.1M H_2SO_4.

Rapid-mix rapid-flow experiments were performed whereby permanganate and quinol phosphate were mixed and passed through the ESR room-temperature cell under conditions producing maximum radical signal at low temperature. The steady-state concentration of radical was examined by slowly scanning the magnetic field in the region of free radical resonance. These experiments also were performed with acid ceric sulfate as oxidant under conditions providing maximum free radical signal at low temperature. With both oxidizing agents chosen, the maximum signal was developed at approximately 100 msec. after mixing. The exact conditions producing these radical signals have been recorded with the illustrations since conditions varied slightly with the quinol phosphate chosen.

ESR spectra of many semiquinones have been reported previously (122). Some of these semiquinone spectra were reconfirmed by dissolving a small sample of quinone in absolute ethanol, diluting with 0.1N $NaOH$, adding less than an equivalent of sodium hydrosulfite and examining at room temperature in the ESR spectrometer. This work was done for general interest and the only semiquinone for which an ESR spectrum had not previously been reported

was that generated from α-tocopheryl quinone.

An attempt was made to produce semiquinone phosphate by electrolytic oxidation of quinol phosphate. A 0.01 M solution of quinol phosphate in pH 11.7 buffer was placed in the electrolytic cell positioned in the microwave cavity. Approximately 5 volts were applied across the calomel reference cell and platinum anode. The magnetic field was scanned in the region of free radical absorption while current flows. The magnetic field also was adjusted to maximum recorder pen displacement while current was flowing, the current was switched off and radical decay was recorded. This experiment was repeated at pH values of 8, 6, 4.5, and 0.8.

An ESR spectrum of the radical formed from U.V. irradiation of frozen 4-hydroxyphenyl-1-phosphate solutions was determined at -165° C. A 0.01 M solution of this quinol phosphate in 0.1 M phosphate buffer, pH 11.7, was frozen in a quartz ESR tube. It was placed in a quartz dewar containing a window, covered with liquid nitrogen and a hydrogen arc source was directed on it for 90 mimutes.

Mitochondria were isolated from rabbit liver by the method of Hogeboom described in Methods of Enzymology (66). A comparison of oxygen consumption in the presence

of NADH or of 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate was made by the technique of Murakami (100) utilizing an oxygen electrode. An ESR spectrum was then obtained for mitochondria treated in the following ways. They were frozen in 0.25 M sucrose and the spectrum obtained at -160°C. They were suspended in a phosphorylating medium with 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate as oxidizable substrate and frozen for low temperature study. They were suspended in a phosphorylating medium in anaerobic tubes, outgassed by repeated evacuation followed by flushing with nitrogen, quinol phosphate was added and the mixture frozen for low temperature study. This last experiment was repeated except the quinol phosphate was replaced by NADH.

M. phlei particles were prepared by the procedure of Brodie (21), suspended in 0.15 M KCl and frozen. For ESR studies, these particles were thawed, suspended in 0.15 M KCl and centrifuged at 4°C. for 90 minutes at 105,000 x g. These particles were resuspended in 0.31 ml of 0.15 M KCl containing 15 μmoles $MgCl_2$, 15 μmoles KF, 40 μmoles K_2HPO_4, 150 μmoles pH 7.7 Tris buffer, 50 γ NAD and 20 γ FAD. In this preparation, the concentration of particles was 200--300 mg protein/ml.

Lyophilized supernatant fraction from M.phlei was dissolved in distilled water to make a solution containing

200 mg protein/ml. This supernatant was divided in half and 80 μmoles vitamin K_1/ml supernatant were suspended in one half by vigorous shaking.

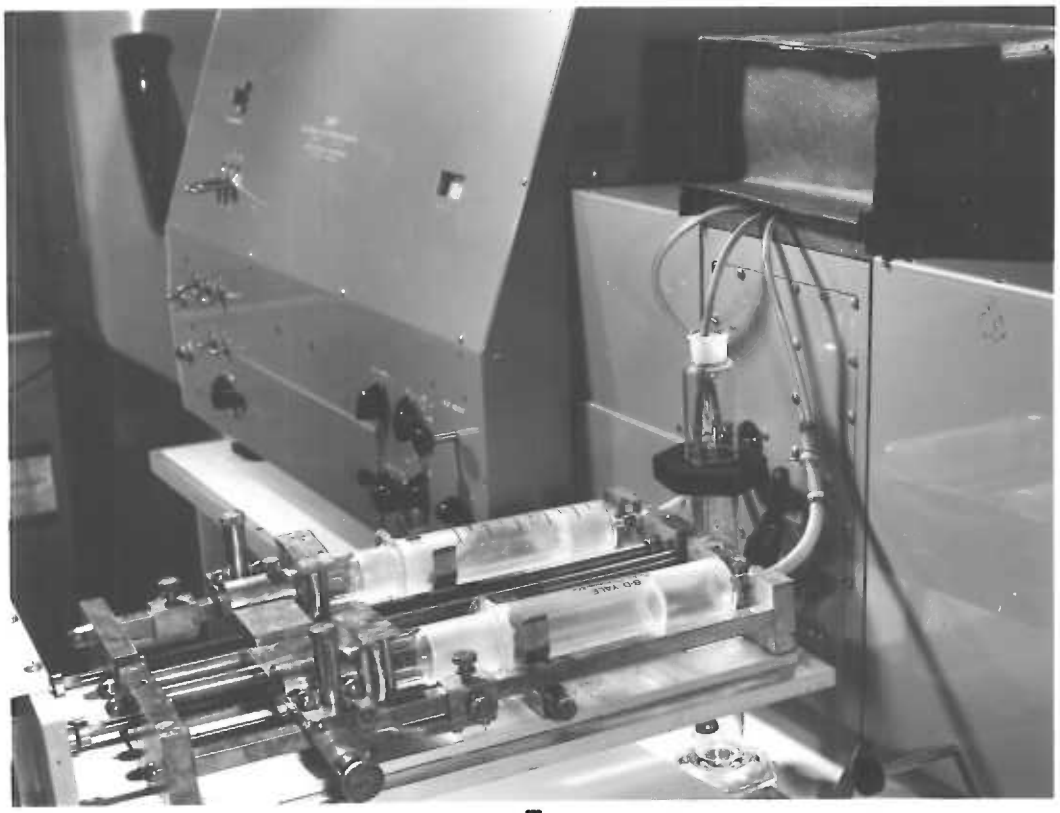
Electron spin resonance spectra were obtained from the particulate and supernatant fractions frozen at -160°C. Equivalent volumes of the suspended particles and supernatant were placed in anaerobic ESR tubes. After repeated evacuations followed by nitrogen flushings, 500 μmoles L-malate/ml was added. This mixture was allowed to incubate for 90 minutes in the dark at room temperature. A second experiment was performed in the same manner except supernatant plus vitamin K was substituted for supernatant alone.

At the end of 90 minutes, 100 μmoles ADP/ml mixture was added and oxygen bubbled through the suspension for 5 minutes. The sample was frozen and an ESR spectrum obtained at -160°C.

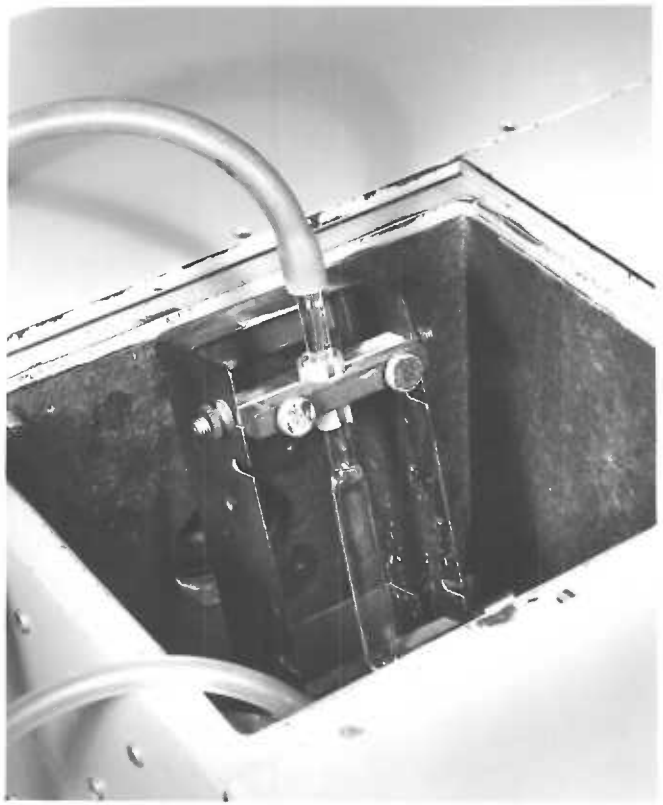
FIG. VII

SYRINGE DRIVE AND FLOW CELL ATTACHMENT FOR THE DETERMINATION OF THE STEADY-STATE ULTRAVIOLET SPECTRUM

This syringe drive, manufactured by Harvard Apparatus Co. is designed to use syringes varying in size from 5--50ml. These may be driven at variable speeds from zero to the maximum by a screw control. It is shown in A connected to a flow cell in the cell compartment. Tubes carrying reagents and exhaust pass through a light-tight box covering the cell compartment. A lucite mixer and attached flow cell is depicted in B, positioned in the spectrometer.



A



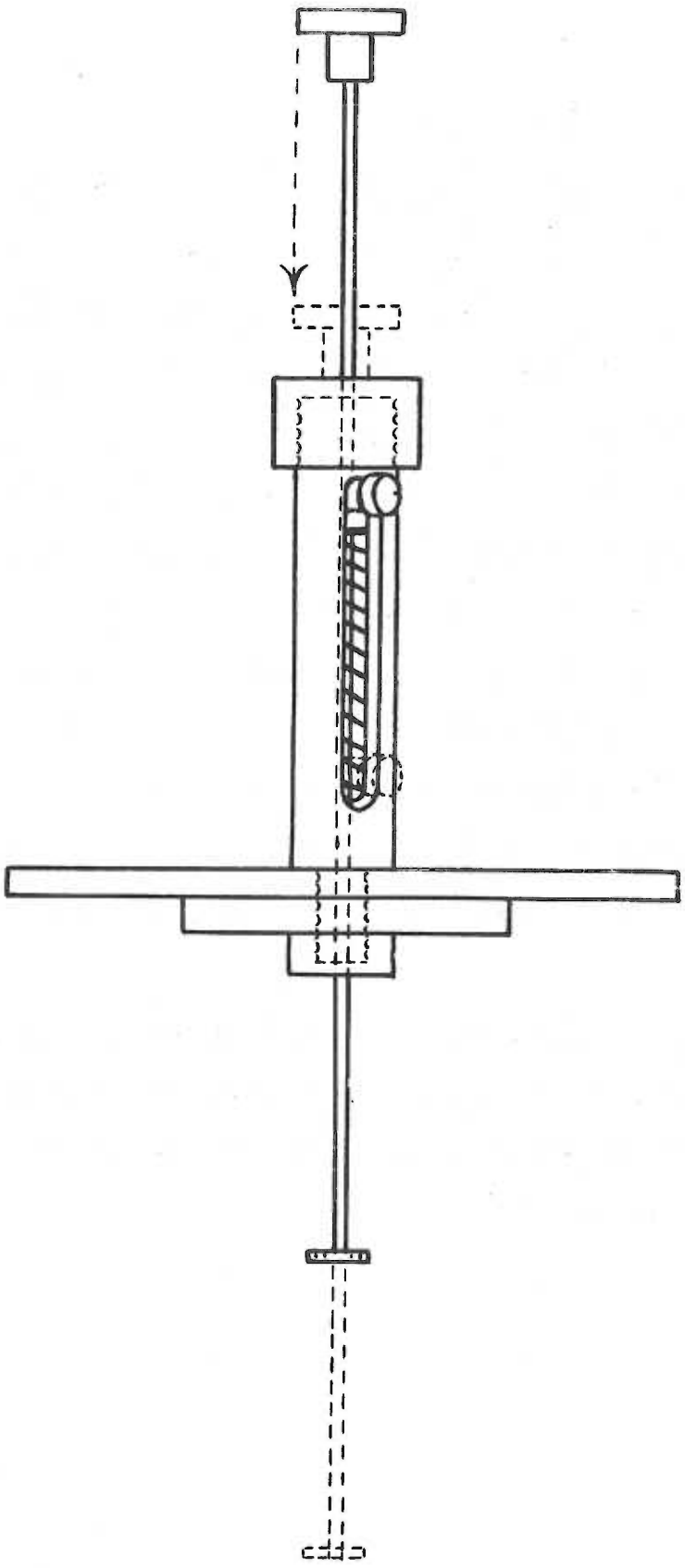
B

FIG. VIII

SPRING LOADED RAPID MIXING DEVICE FOR SPECTROPHOTOMETER CELLS

This device is attached to the cell compartment cover. A small diameter steel rod extends through the compartment cover with a small perforated plastic plate at its lower end and a knurled metal cap at its upper end. The perforated plastic plate just fits inside a 1 x 1 cm cuvette. For use, a small volume of enzyme solution is placed on the plastic plate and the cell compartment closed. At a given time, this plate is forced into the substrate solution by depressing the knurled knob at the top of the steel rod. A spring retrieves the unit. Solution passing through the perforations of the plate rapidly mix enzyme with substrate and the absorption may be followed very soon after mixing.

This device was designed by Dr. T. Yamano and Dr. H. S. Mason of the Biochemistry Department, and was fabricated by Mr. J. Yowell of the Research Instrument Service, University of Oregon Medical School.



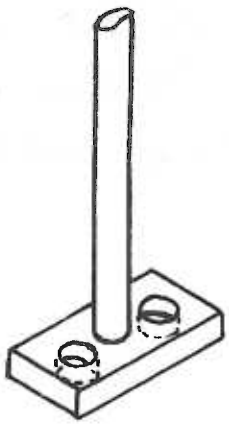


FIG. IX

ESR CELLS

Tube A consists of a 3 mm I.D. quartz tube in which frozen aqueous solutions may be examined at low temperature. Tube B, shown attached to a lucite mixer, composes the Varian V4549 liquid flow unit. Aqueous reactant solutions may be passed through the lucite mixer and attached ESR cell at variable speeds from zero to an undetermined maximum, while undergoing examination by ESR at room temperature. Tube C has a glass top of about 2 cm I.D. fused to a quartz base of the same dimensions as A. It is used by filling with isopentane, immersing in liquid nitrogen and injecting a fine stream of sample that is very rapidly frozen. Frozen sample particles are forced to the bottom of C by the tamper unit beside it. Tube D is an electrolytic cell designed to allow ESR spectroscopy while aqueous solutions are electrolysed. Cell E is designed for anaerobic studies. The samples are placed in the base of tube E, which is identical to A, the atmosphere is adjusted by repeated evacuation followed by flushing with N_2 through the side arm and the sample is frozen for examination at low temperature. A high incidence of tube breakage forced the fabrication of the two piece unit, E, shown. A standard taper joint connects the top to a 3mm inside diameter quartz tube. This unit was fabricated by Mr. G. Weiss of Electro-glass Laboratories.

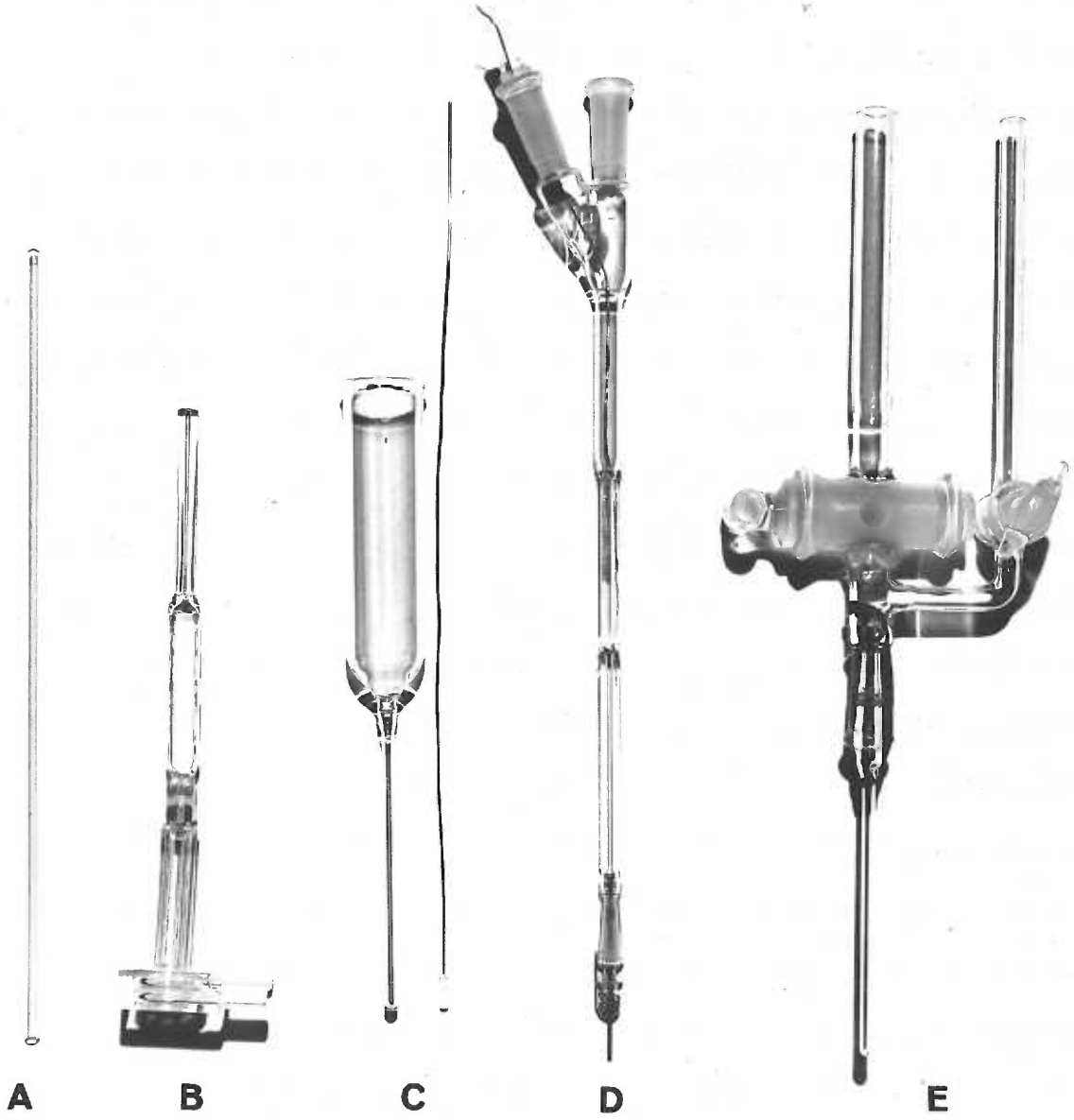

A
B
C
D
E

FIG. X

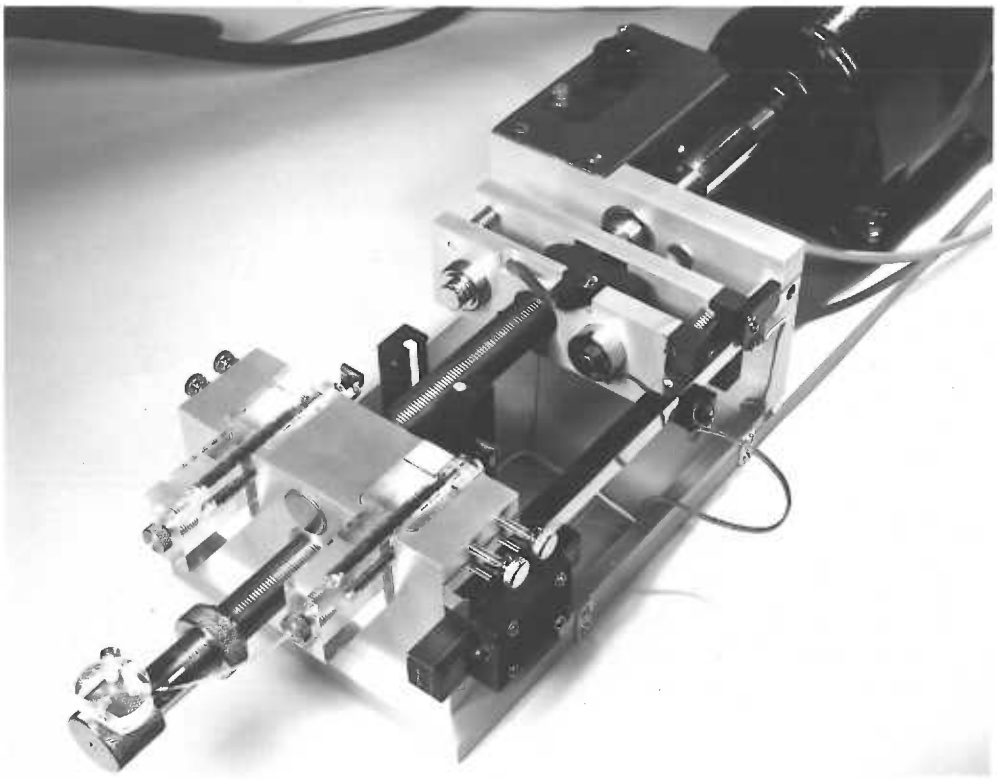
RAPID-MIX RAPID-FREEZE SYSTEM

This apparatus* consists of a motor driven set of syringes, a mixer, an ejection nozzle and a system for rapidly freezing the effluent. It is driven by a variable-speed Servo-Tec motor with control units. Precision 1 ml Hamilton syringes are mounted in lucite blocks and are connected to a lucite mixer by polyethylene tubing. Effluent from the mixer passes through a short length of P.E.#10 polyethylene tubing and is injected into cold isopentane.

A monitoring system is composed of a linear potentiometer, contact switch and Tektronix 535A oscilloscope. Contact between the syringe drive and syringe plunger triggers a horizontal tracing on the oscilloscope which moves at a controlled rate across the screen. The vertical position of this trace is determined by a linear potentiometer mounted beside one of the syringes. It and the syringe plungers are effected simultaneously by the syringe driving mechanism. A polaroid photograph of the oscilloscope trace allows the volume of effluent as well as the expulsion time to be determined. The entire unit is illustrated in A and a close-up of the syringes, potentiometer and mixer is shown in B.

* This device was designed by Drs. T. Yamano and H. S. Mason of the Biochemistry Department and fabricated by G. Johnston and J. Yowell of the Research Instrument Service, University of Oregon Medical School.

A

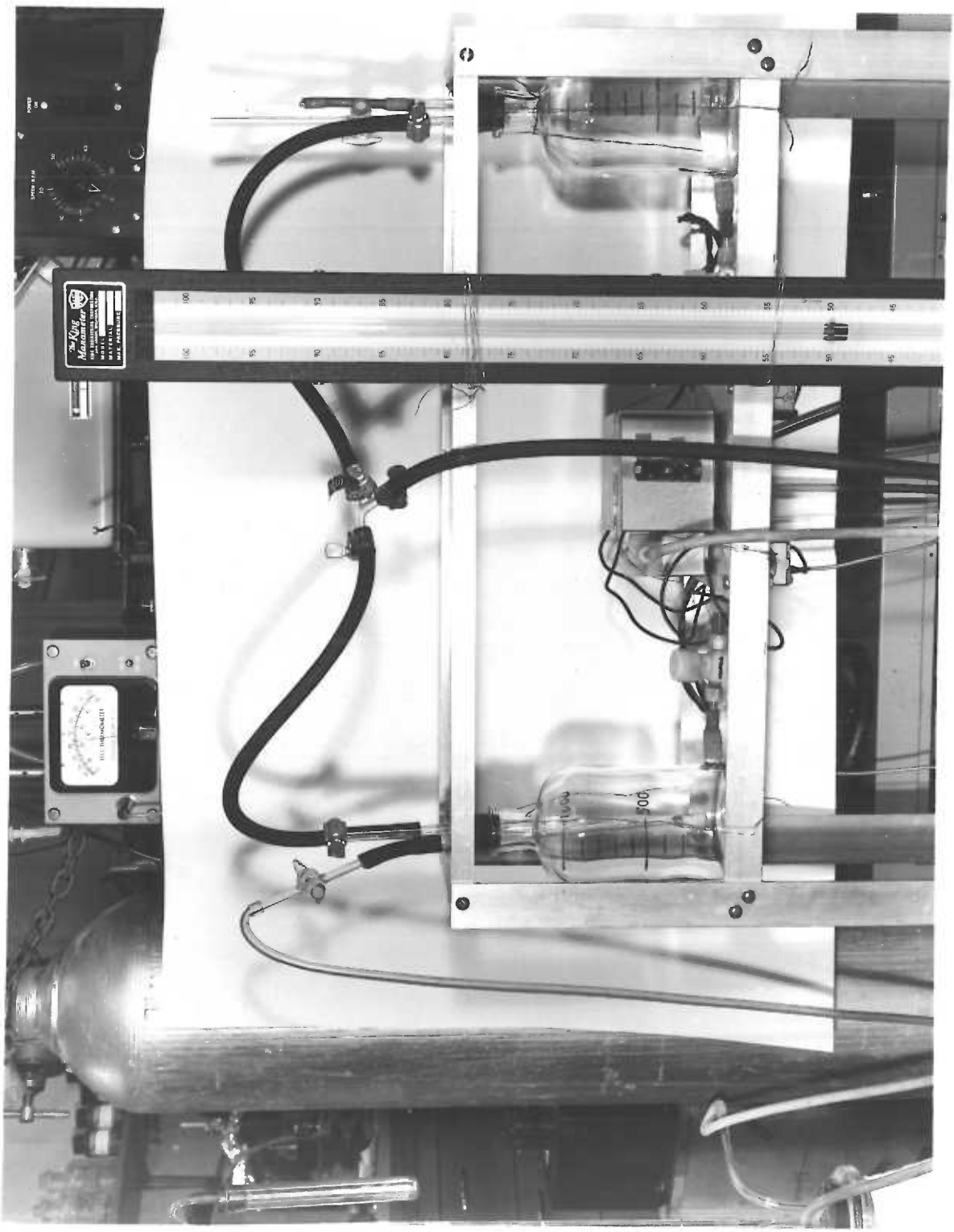


B

FIG. XI

PNEUMATIC FLOW SYSTEM

By this device, the flow of reagent through the mixer and ESR cuvette is controlled by air pressure. The pressure is maintained by a large reservoir and measured with a manometer. In practice, the pressure is adjusted with the reservoir attached to each of two reagent bottles. At a given time, solonoid valves mounted under the chassis, open and allow reagents to flow from both vessels at the same rate. These reagents pass through tygon tubing to a lucite mixer attached to the ESR cell (Fig. IX B). After passing through the ESR cavity where measurements are made, the mixture passes through another tygon tube to a product trapping reservoir.


The King Manometer
MATERIAL
MAX. PRESSURE

III RESULTS

1. SPECTROPHOTOMETRIC RESULTS

It was observed that the spectrum of 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate differed markedly from that of the corresponding quinone (Fig. XII). In aqueous solution, the quinone spectrum was quite different from that reported for solutions in hexane (45) or alcohol (49). The maxima were shifted toward the red and one at 260 mμ was missing entirely. Our spectrum in hexane solution agreed with that reported earlier (45).

When this quinol monophosphate was oxidized by peroxidase-H_2O_2 and the reaction system extracted with hexane, a yellow product having an identical U.V. spectrum to that reported earlier (45) was obtained. Removal of hexane under vacuum left a yellow crystalline solid which melted with decomposition between 118.5 - 121°C. Comparison of its I.R. spectrum with that from an authentic sample of 2,3-dimethylnaphthoquinone revealed no detectable differences.

Peroxidase-H_2O_2 rapidly converted 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate to 2,3-dimethylnaphthoquinone at pH 4.5. The maximum rate of change of substrate concentration was 4 μM sec-1 (Fig. XIII). Increasing the substrate concentration led to precipitation of quinone,

which obscured the U.V. spectrum and made kinetic study impossible. It was not economically feasable to increase peroxidase concentration and increases in peroxide, without corresponding increases in substrate concentration, decreased the oxidation rate. Later experiments indicated small decreases in initial rate occurred if the H_2O_2 concentration was lowered but, at 10^{-4} M, the marked rate change during reaction was avoided.

Since the extinction coefficients at the maxima for both substrate and product were known, it was possible to determine the concentration of both as a function of time by solving simultaneous equations. In both acid and alkaline pH, the percent substrate changed was very nearly equal to the percent product formed (Fig. XIV) at any time. The absorption properties of quinone under the conditions of this experiment did not remain constant over appreciable periods of time. In some instances, a faint precipitate appeared on long standing suggesting that absorption measurements were not made on a true solution.

The rates at which 4-hydroxy-2-methylnaphthyl-1-phosphate and 4-hydroxynaphthyl-1-phosphate were oxidized by peroxidase in acid solution were much lower than for the 2,3-dimethyl derivative. In alkaline solution, the

rates were sufficiently slow that non-enzymatic oxidation contributed heavily to the overall reaction. For this reason, no attempts were made to analyse the data from these oxidations. Table I lists some substrates and relative rates at which they are oxidized by peroxidase-H_2O_2. No apparent increase in reaction rate was caused by 10^{-3} M Mg^{++} ions at pH 5.1.

TABLE I

PEROXIDATIC OXIDATION OF QUINOL PHOSPHATES

Substrate	Relative Rate
4-hydroxy-2,3-dimethylnaphthyl-1-phosphate	fast
4-hydroxy-2-methylnaphthyl-1-phosphate	slow
4-hydroxynaphthyl-1-phosphate	slow
α-tocopheryl phosphate	nil
6-chromanyl phosphate of vitamin K_1	slow
2-hydroxyphenyl-1-phosphate	fast
4-hydroxyphenyl-1-phosphate	fast

2. ELECTRON SPIN RESONANCE RESULTS

Examination of 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate solutions oxidized by peroxidase-H_2O_2, under conditions found optimal in spectrophotometric experiments and frozen shortly after mixing, revealed no detectable free radical species. No detectable radical was found when this experiment was repeated at pH8. However, when reactant solutions were frozen under optimal conditions approximately 20 msec. after mixing, by means of the rapid-mix rapid-freeze apparatus, a low concentration of radical was detected. This speed represented the maximum attainable in our apparatus. Lowering speed led to a decrease in this radical concentration. A change in reactant concentration or pH caused either no change or a decrease in radical concentration and no radical species could be detected when this reaction was studied at room temperature by means of a rapid-mix rapid-flow system.

This quinol phosphate, placed on a TEAE-cellulose column at pH 4.5 and oxidized by passing peroxidase and H_2O_2 over the column, formed a red band which changed to yellow within a few seconds. Examination of the red substance at -160°C revealed no radical species. The yellow compound had a similar but distinctly different U.V.

spectrum from that of 2,3-dimethylnaphthoquinone. A flow experiment by which quinol phosphate was oxidized on a TEAE-cellulose column with peroxidase-H_2O_2, while the column was positioned in the ESR cavity, gave a small concentration of some radical species. Oxidation of 4-hydroxy-2-methylnaphthyl-1-phosphate on a TEAE-cellulose column produced similar results. A red band developed rapidly which changed to yellow within 30 seconds. This yellow substance did not have the same U.V. spectrum as 2-methylnaphthoquinone. 4-Hydroxynaphthyl-1-phosphate was oxidized at an insignificant rate on the column.

Oxidation of 2-hydroxyphenyl-1-phosphate by peroxidase-H_2O_2 at pH 4.5 caused a darkening of the solution. ESR spectroscopy revealed a small concentration of some stable radical species.

The 6-chromanyl phosphate of vitamin K_1 was slowly oxidized by peroxidase-H_2O_2 under conditions found optimal for quinol phosphates. ESR spectroscopy of solutions allowed to oxidize for 30 msec. before freezing indicated a free radical intermediate but its concentration was too low for identification. A similar experiment with α-tocopheryl phosphate found no oxidation occurring.

Several strong oxidizing agents were found to oxidize quinol phosphates readily to their corresponding quinones

but the reaction rate or optimum reaction conditions could not be predicted. Hypobromite oxidized α-tocopheryl-phosphate and 4-hydroxy-2-methylnaphthyl-1-phosphate rapidly, producing a radical intermediate. In the latter case, a doublet ESR signal was observed at -160°C and high amplification. Chlorine oxidized α-tocopheryl phosphate via a radical intermediate but radical concentration was too low for an identification to be made. Chloranil was found an effective oxidizing agent but its semiquinone obscured any other radical signal generated. Ceric sulfate in acid solution produced radical intermediates from 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate; 4-hydroxy-2-methylnaphthyl-1-phosphate; 4-hydroxynaphthyl-1-phosphate; α-tocopheryl phosphate and the 6-chromanyl phosphate of vitamin K_1. Only a small radical signal, devoid of hyperfine structure, was detected from the quinol phosphates. A broad doublet signal was detected from both α-tocopheryl phosphate and the 6-chromanyl phosphate of vitamin K_1. No additional hyperfine structure was present in these ESR signals even at room temperature and at low modulation amplitudes. These radicals decayed rapidly with an apparent half-life of a few seconds.

Alkaline permanganate was found to oxidize all quinol phosphates at a rapid rate and chromanyl phosphates at a

slow rate. Examination of chromanyl phosphate solutions undergoing alkaline permanganate oxidation failed to reveal a detectable free radical. However, quinol phosphates oxidized by alkaline permanganate produced high concentrations of radical intermediate. This concentration approaches 10^{-4} M from 10^{-2} M quinol phosphate. Table II lists a group of quinol phosphates and the concentration of radical detected during their oxidation. These radicals decayed relatively slowly; an apparent half life of a few seconds being the rule. In every experiment, the radical signal detected was obviously different from the signal observed from the corresponding semiquinone. In addition, a doublet was present in these signals with varying degrees of prominence. This doublet assumed an unusually large splitting of approximately 20 gauss when positions ortho to the phosphate group were substituted. Hyperfine structure was superimposed on this doublet signal in some instances.

TABLE II

DETECTED CONCENTRATIONS OF SEMIQUINONE PHOSPHATES

Quinol Phosphate	Radical Concentration
4-hydroxy-2,3,5,6-tetramethylphenyl-1-phosphate	6.3×10^{-5} M
4-hydroxy-2,3-dimethylnaphthyl-1-phosphate	5.4×10^{-4} M
α-tocopheryl phosphate	1.5×10^{-4} M
4-hydroxy-2-methylnaphthyl-1-phosphate	7.5×10^{-5} M
4-hydroxynaphthyl-1-phosphate	2.0×10^{-5} M
4-hydroxyphenyl-1-phosphate	1.7×10^{-5} M
2-hydroxyphenyl-1-phosphate	1.6×10^{-5} M

Electrolytic oxidation failed to produce a detectable concentration of free radical except at pH 11.7. At this pH, radical concentration was not sufficiently high for a positive identification to be made. However, the fine structure of the signal produced when 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate was oxidized electrolytically corresponded to the fine structure observed during its chemical oxidation. As electrolysis progressed, stable radical species became increasingly prevalent. Consequently, radical spectra obtained by this method were unreliable.

Irradiation of a frozen 0.01 M solution of 4-hydroxyphenyl-1-phosphate with ultraviolet light produced a free radical whose ESR signal differed slightly from the chemi-

cally produced signal.

From measurements of oxygen consumption, it appeared that 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate was oxidized almost as readily as NADH by rabbit liver mitochondria. However, this quinol phosphate consumed oxygen at only a slightly lower rate when incubated in the physiological medium minus mitochondria. Autoxidation progressed rapidly in the physiological medium.

Electron spin resonance spectra were obtained from mitochondria in 0.25 M sucrose, from mitochondria incubated fifteen minutes aerobically in a phosphorylating media plus 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate, from mitochondria incubated anaerobically for fifteen minutes in a phosphorylating medium plus 4-hydroxy-2,3-dimethynaphthyl-1-phosphate, and from mitochondria incubated anaerobically for fifteen minutes in a phosphorylating medium plus NADH. Only with NADH was there a significant change in ESR spectrum.

Results of experiments with M phlei fractions were inconclusive. The ESR spectrum of active phosphorylating fractions is altered by anaerobic incubation with vitamin K_1 but it is not clear what these changes mean.

ANALYSIS OF PRODUCTS

Paper chromatography of reaction solutions indicated several soluble phosphate containing compounds were present. Ortho phosphate and pyrophosphate were used as reference spots on the chromatograms. Of the spots from unknowns, ortho phosphate and pyrophosphate were identified by their positions. However, other phosphate containing spots were present. Table III summarizes the results of chromatographic analysis.

TABLE III

CHROMATOGRAPHIC ANALYSIS OF

PHOSPHATE CONTAINING COMPOUNDS

Solution Analysed	Oxidant	R_f of Detected Spot
Oxidized α-tocopheryl phosphate	$Ce(SO_4)_2$ H_2SO_4	0.2, 0.3, 0.6, 0.7, 0.9
Control Solution $Na_2P_2O_7$, Na_2HPO_4	--------	--- 0.3, ---- 0.7, ---
. . . . .	. . . . .	. . . . .
Oxidized 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate	Turnip-peroxidase H_2O_2, pH 4.5	-- 0.4, ---- 0.7, 0.9
Oxidized 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate	$KMnO_4$ pH 10.95	--- 0.4, ---- 0.7, ---
Control Solution $Na_2P_2O_7$, Na_2HPO_4	--------	--- 0.4, ---- 0.7, ---

All quinone products were not analysed. However, a yield of 92% was obtained in the conversion of 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate to 2,3-dimethylnaphthoquinone by alkaline permanganate. An almost quantitative conversion was observed for this conversion when peroxidase-H_2O_2 was the oxidant. Acid ceric sulfate oxidation of α-tocopheryl phosphate led to a 77% yield of tocopheryl quinone.

FIG. XII

The U.V. spectrum of 2,3-dimethylnaphthoquinone is represented by (——) and that of 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate, by (- - - - -). Both spectra have been determined on 10^{-4} M solutions at pH 4.5 in 0.1 M acetate buffer.

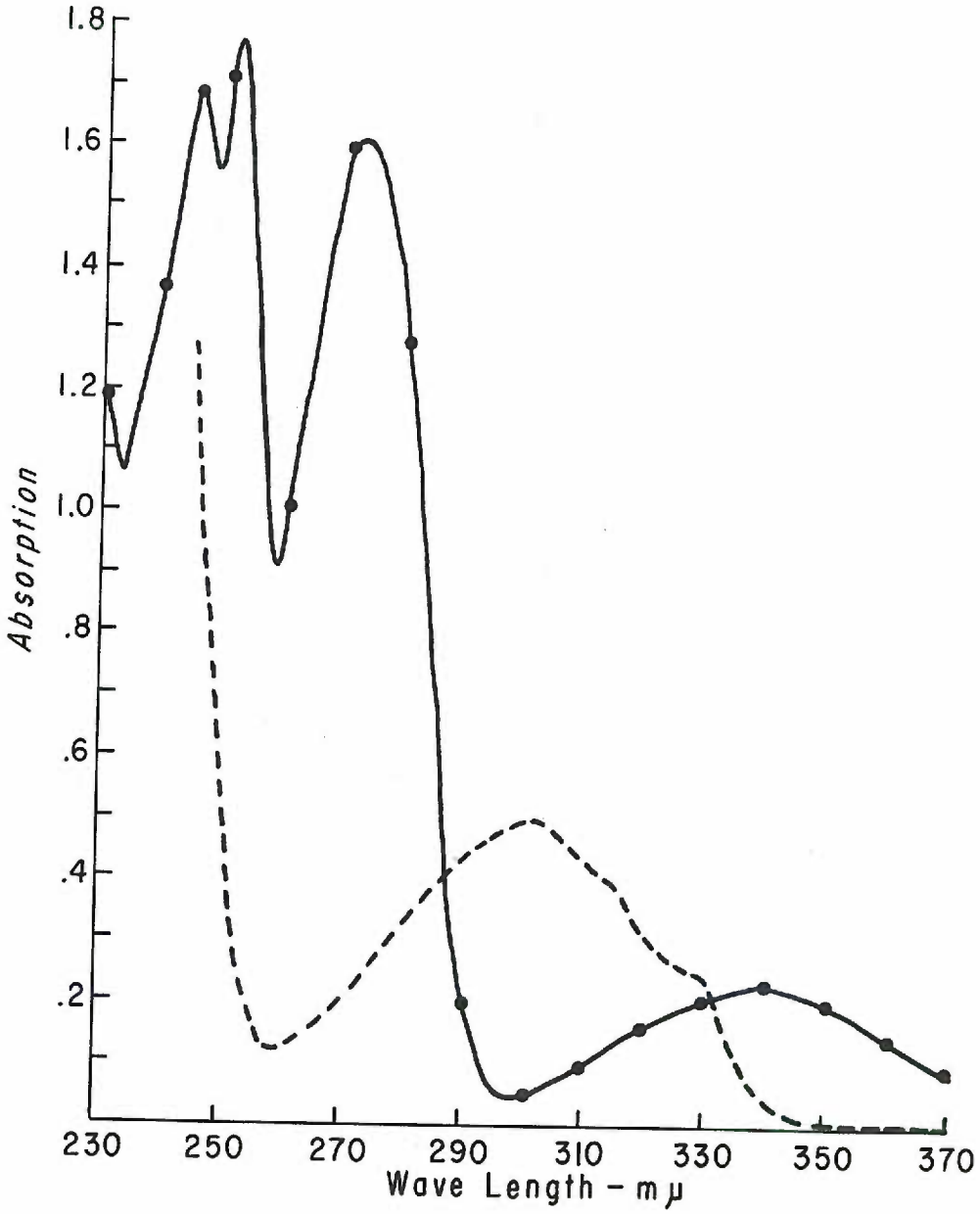

1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
Absorption
230
250
270
290
310
330
350
370
Wave Length - mμ

FIG. XIII

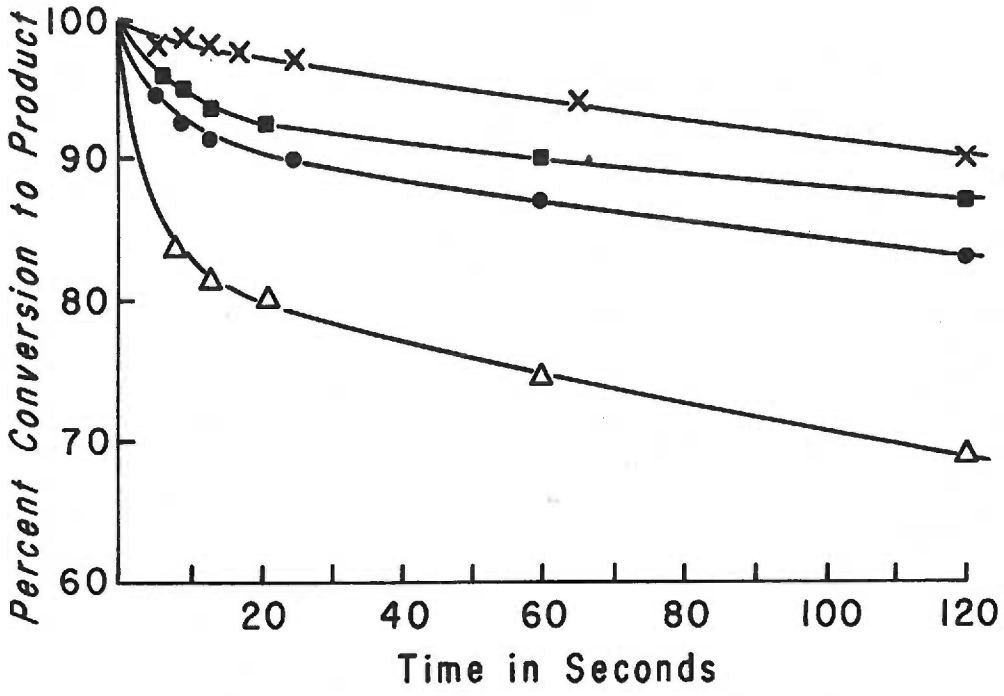
EFFECT OF pH ON THE RATE OF PEROXIDATIC OXIDATION OF 2,3-DIMETHYLNAPHTHOQUINOL PHOSPHATE

Percentage conversion of 2,3-dimethylnaphthoquinol phosphate to its oxidized product is recorded as a function of time. The reaction mixture is composed of 10^{-2} M H_2O_2, 5 x 10^{-7} M turnip peroxidase and 10^{-4} M quinol phosphate. Various reaction pH's are represented in the figure by △ for pH 4.5, ⊙ for pH 5.1, ⊡ for pH 7.1 and X for pH 8.5.

FIG. XIV

RATES OF QUINOL PHOSPHATE DISAPPEARANCE AND QUINONE APPEARANCE AS A FUNCTION OF pH

Recorded are concentrations of 2,3-dimethylnaphthoquinol phosphate reactant and 2,3-dimethylnaphthoquinone product at various times after initiation of oxidation by peroxidase and H_2O_2. Initial concentrations of these compounds are 10^{-4} M quinol phosphate, 10^{-2} M H_2O_2 and 5 x 10^{-7} M peroxidase. Concentration of quinolphosphate at pH 4.5 is represented by ⊡ and by ⊙ at pH 8.5. Quinone concentration is represented by △ at pH 4.5 and by X at pH 8.5.


Percent Conversion to Product
100
90
80
70
60
20
40
60
80
100
120
Time in Seconds

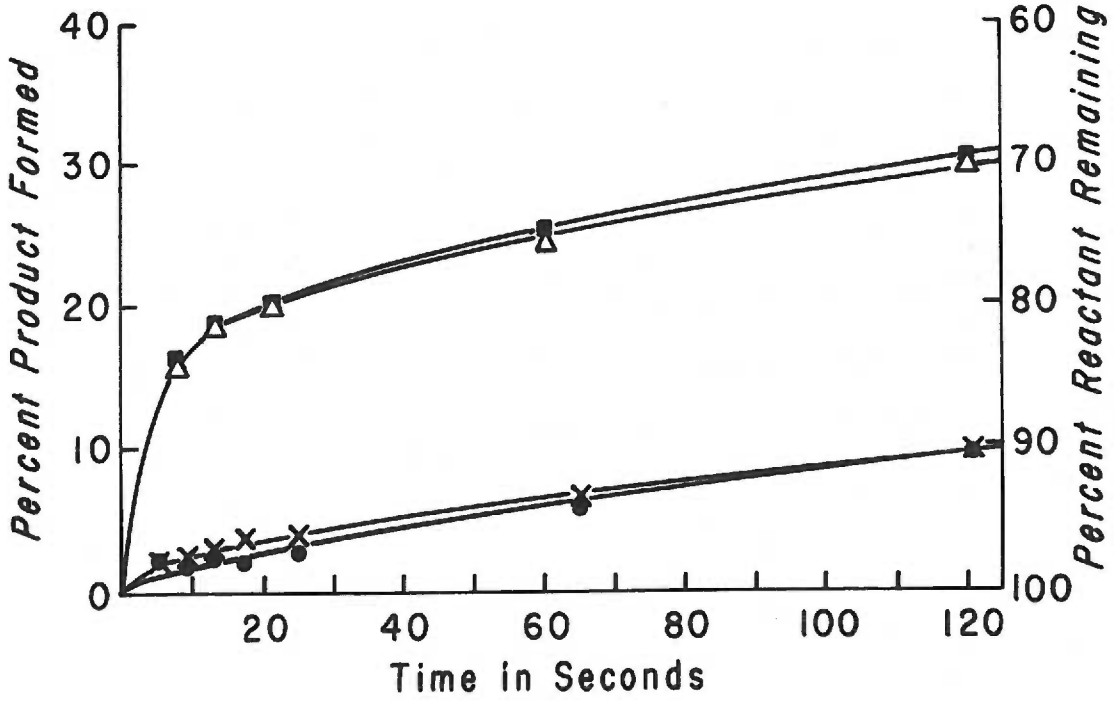

Percent Product Formed
40
30
20
10
0
Percent Reactant Remaining
60
70
80
90
100
20
40
60
80
100
120
Time in Seconds

FIG. XV

RADICAL INTERMEDIATE IN THE OXIDATION OF α-TOCOPHERYL PHOSPHATE

The ESR derivative curve recorded was obtained from ceric sulfate oxidation of α-tocopheryl phosphate in acid solution at 25°C, 58 msec. after mixing the reagents. A 0.02 M ceric sulfate solution in 0.1 M H_2SO_4 was mixed with an emulsion of α-tocopheryl phosphate and passed through the ESR cavity by means of a rapid flow apparatus. Sufficient α-tocopheryl phosphate to prepare a 0.01 M solution was emulsified in 0.1 M H_2SO_4, containing 1% sodium dodecyl sulfate.

A broad doublet signal is obtained which contains no hyperfine structure. Included is a graphically integrated ESR absorption spectrum.

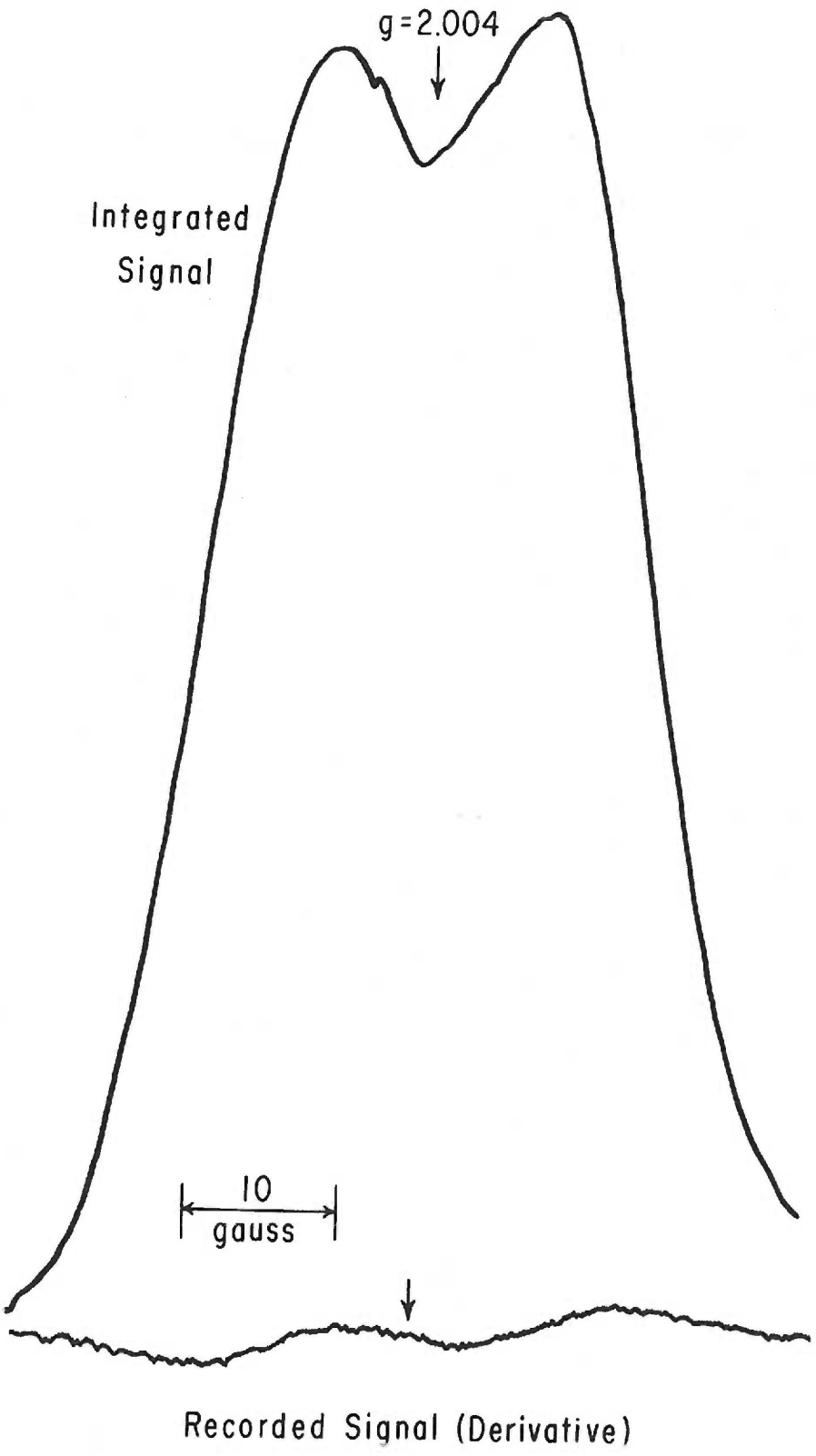

g=2.004
Integrated
Signal
10
gauss
Recorded Signal (Derivative)

FIG. XVI

RADICAL INTERMEDIATE IN THE OXIDATION OF THE 6-CHROMANYL PHOSPHATE OF VITAMIN K_1

Sufficient 6-chromanyl phosphate of vitamin K_1 to prepare a 0.01 M solution, was emulsified in 0.1 M H_2SO_4 containing 1% sodium dodecyl sulfate. The recorded ESR derivative signal was obtained by mixing this emulsion with an equivalent volume of 0.02 M ceric sulfate in 0.1 M H_2SO_4, freezing 40 msec. after mixing and examining at -160°C. with the ESR spectrometer.

FIG. XVII

α-TOCOPHERYL SEMIQUINONE ESR SPECTRUM

The ESR derivative signal recorded here was obtained from a dilute solution of α-Tocopheryl quinone in alkaline ethanol to which half an equivalent of sodium dithionite had been added. This room temperature spectrum contains 17 discernible lines and broad absorption at the extremities suggests 4 more lines.

Fig. XVI

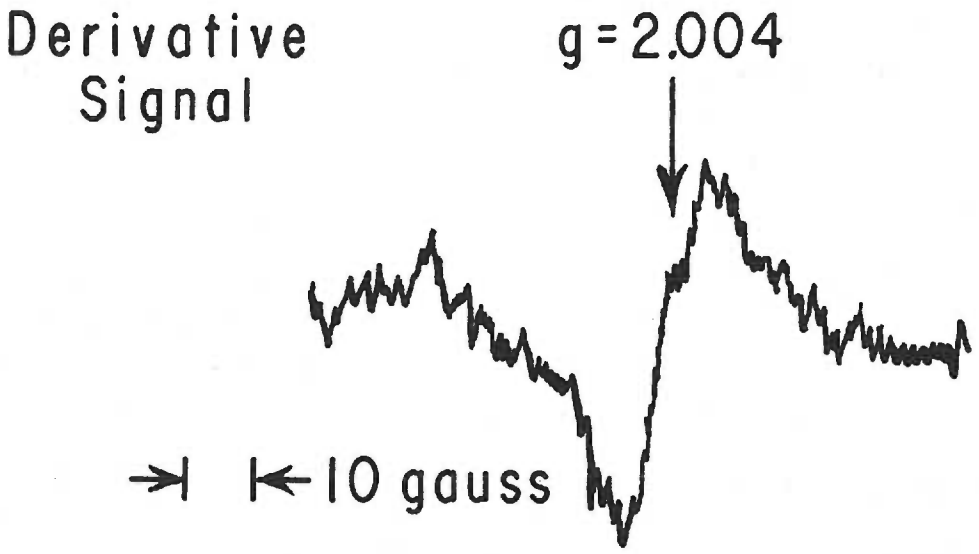


Fig. XVII

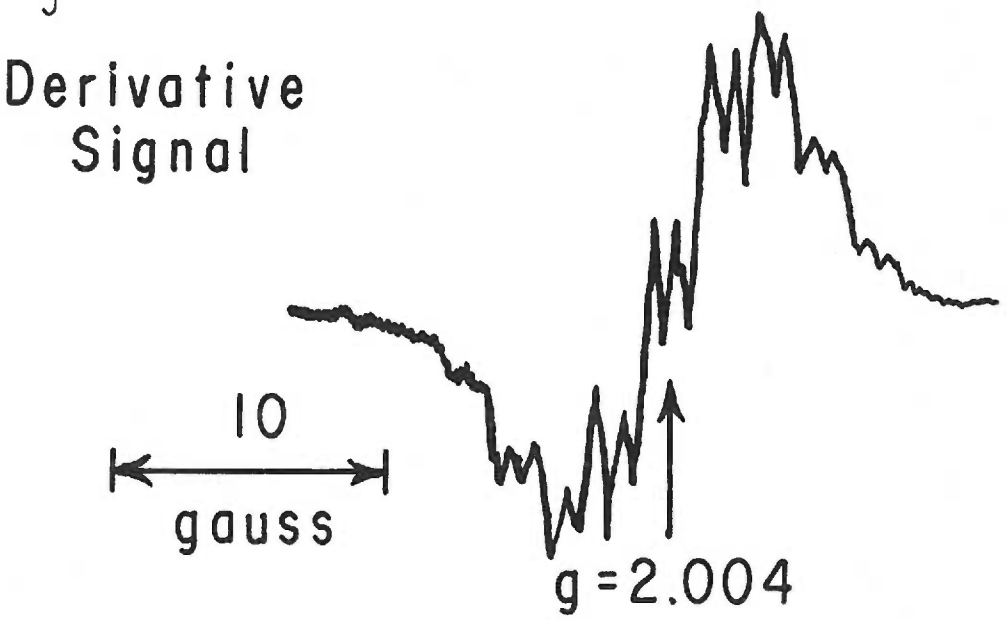


FIG. XVIII

RADICAL INTERMEDIATE IN THE OXIDATION OF 4-HYDROXY-2,3,5,6-TETRAMETHYLPHENYL-1-PHOSPHATE

Recorded is the ESR signal obtained from a steady-state concentration of radical formed during a flow experiment at approximately 25°C. This radical was observed 90 msec. after mixing 0.01 M 4-hydroxy-2,3,5,6-tetramethylphenyl-1-phosphate with an equal volume of 0.006 M $KMnO_4$. Both solutions were prepared in a 0.1 M phosphate buffer at pH 11.7. The derivative signal observed contains an apparent 50 lines.

A graphic integration of this recorded signal presents an ESR absorption spectrum with a 10 line fine structure and a g value of 2.004. The 10 major lines are in the ratio of 1:6:15.7:20.4:19.5:19.5:20.4:15.8:6:1.

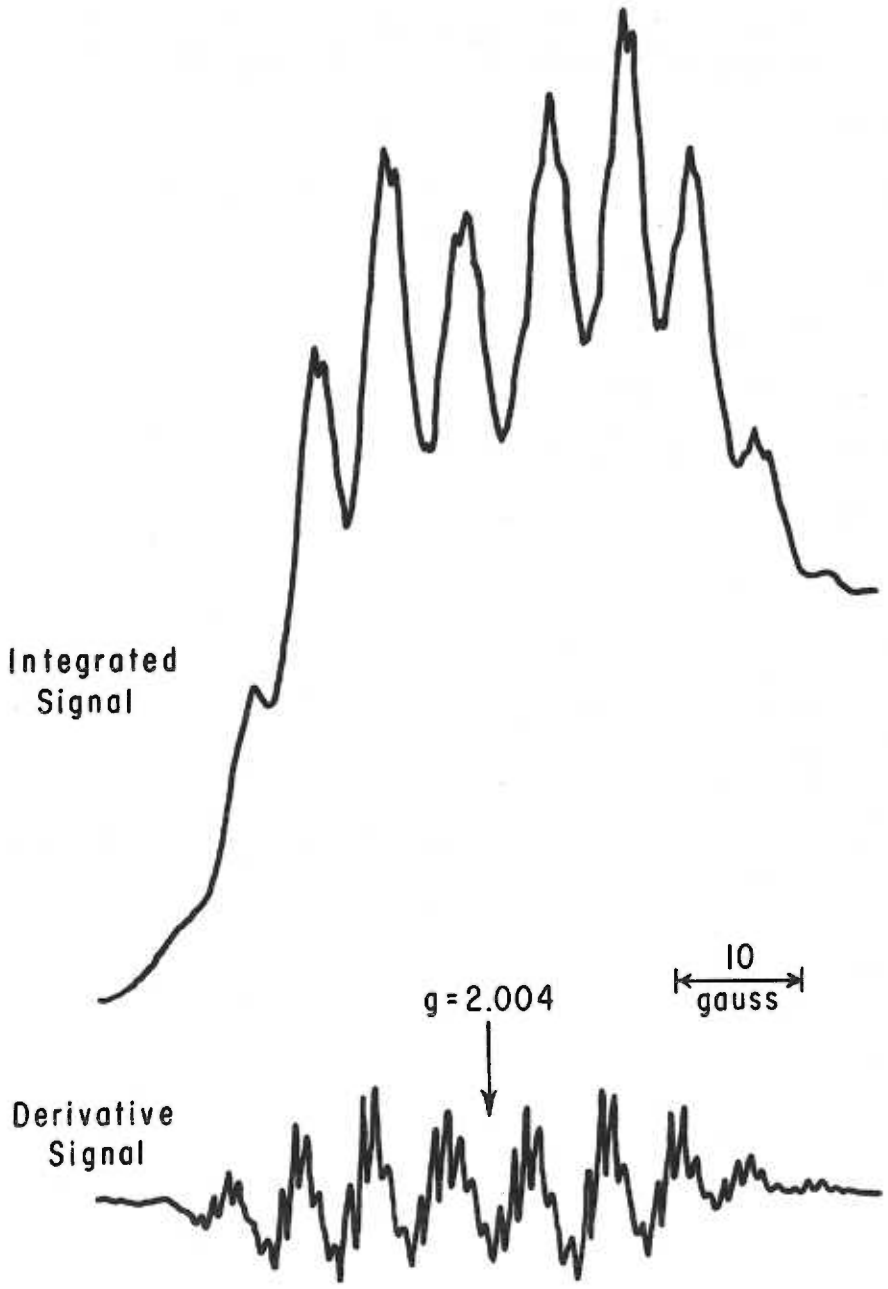

Integrated
Signal
10
gauss
g = 2.004
Derivative
Signal

FIG. XIX

RADICAL INTERMEDIATE IN THE OXIDATION OF
4-HYDROXY-2,3-DIMETHYLNAPHTHYL-1-PHOSPHATE

The recorded derivative signal was obtained from a steady-state radical concentration during an ESR flow experiment. The radical was studied as reactant solution passed through a flow cell 140 msec. after mixing at approximately 25°C. The initial reaction mixture was composed of 0.017 M 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate, 0.006 M $KMnO_4$ and 0.55 M Na_2CO_3. The reaction pH was 10.95.

The integrated signal was derived manually from the recorded signal. In this integrated signal, a spectrum of six major lines in the approximate ratio of 1:3:4:4:3:1 is apparent. The g value was 2.004.

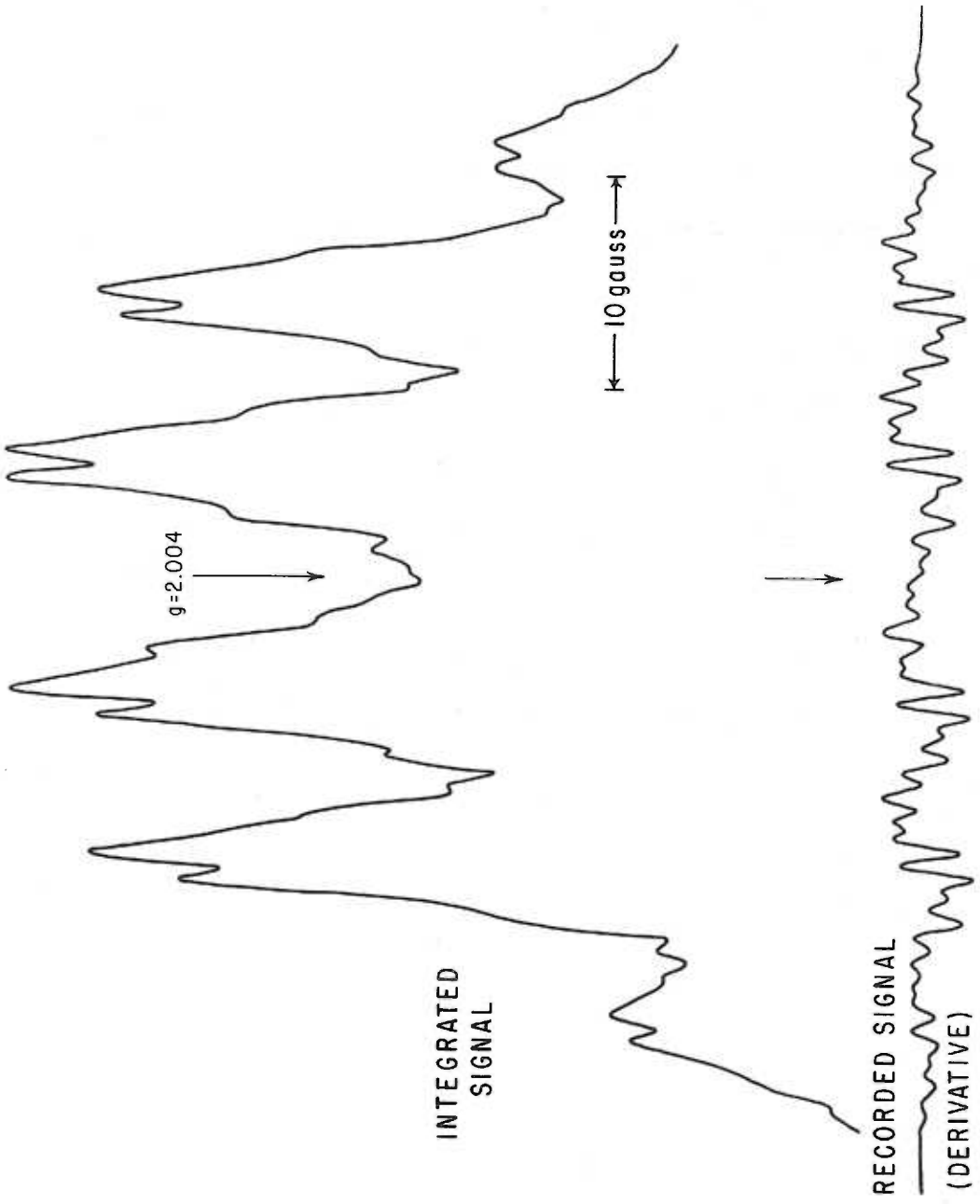

g=2.004
10 gauss
INTEGRATED SIGNAL
RECORDED SIGNAL
(DERIVATIVE)

FIG. XX

RADICAL INTERMEDIATE IN THE OXIDATION OF
4-HYDROXY-2-METHYLNAPHTHYL-1-PHOSPHATE

Recorded is the observed derivative curve when 0.005 M 4-hydroxy-2-methylnaphthyl-1-phosphate was oxidized by 0.003 M $KMnO_4$ in 0.55 M Na_2CO_3 at approximately 25°C and pH of 10.95. The recorded signal was obtained from the steady-state concentration of radical produced 165 msec. after reactants were mixed by means of a rapid-mix rapid-flow device.

This signal is composed of a pair of triplets separated by approximately 18 gauss. Under reaction conditions only the triplet hyperfine structure is resolved. A graphically integrated ESR absorption spectrum is included.

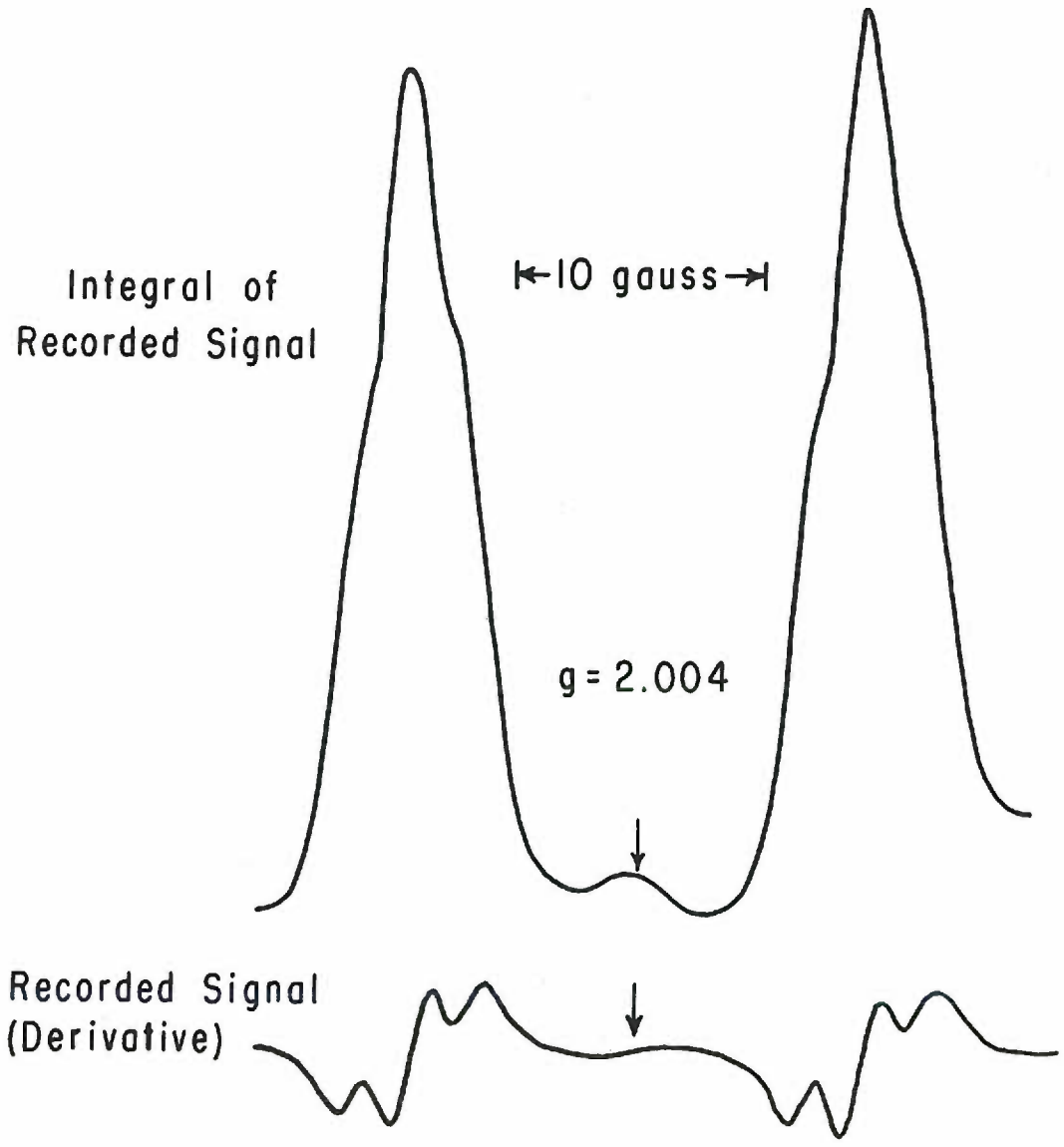

Integral of
Recorded Signal
10 gauss
g = 2.004
Recorded Signal
(Derivative)

FIG. XXI

RADICAL INTERMEDIATE IN THE OXIDATION OF
4-HYDROXY-3-CHLORO-2-METHYLNAPHTHYL-1-PHOSPHATE

Recorded is the observed derivative signal when a 0.013 M solution of 4-hydroxy-3-chloro-2-methylnaphthyl-1-phosphate was oxidized by 0.008 M $KMnO_4$ at pH 11.7 and 25°C. Equivalent volumes of reagent solutions were mixed and passed through the ESR flow cell at a rate such that 47 msec. passes from mixing until the steady-state concentration was examined.

Included is a graphically derived ESR absorption spectrum.

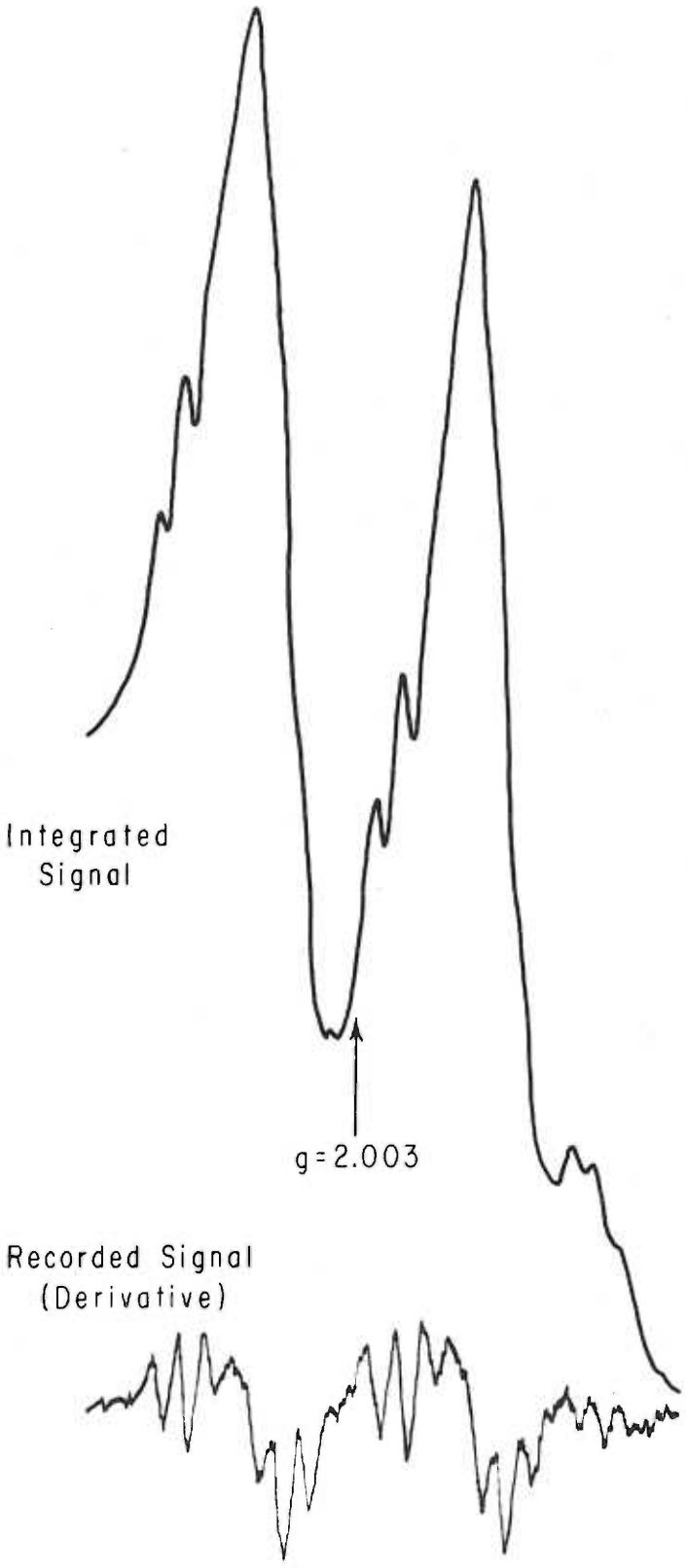

Integrated
Signal
g=2.003
Recorded Signal
(Derivative)

FIG. XXII

RADICAL INTERMEDIATES IN THE OXIDATION OF 4-HYDROXY-2,3,5,6-TETRACHLOROPHENYL-1-PHOSPHATE

In part (A), is recorded the observed derivative signal and derived absorption spectrum from a solution composed of equal parts 0.01 M 4-hydroxy-2,3,5,6-tetrachlorophenyl-1-phosphate and 0.006 M $KMnO_4$. Both reactants were prepared in 0.1 M phosphate buffer at pH 11.7. This solution was allowed to react for about 15 seconds after mixing and then frozen. The ESR spectrum was determined at -160°C. Only a doublet signal with a g value of 2.006 and a 15.2 gauss splitting is observed.

Examination of the ESR spectrum determined about 15 seconds after mixing at room temperature revealed no radical signal. The free radical portion of the spectrum is reproduced in (B). However, about fifty seconds after reactants have been mixed, a radical species (C) becomes apparent in the room temperature spectrum. This radical contains only a simple singlet absorption with a g value of 2.006.

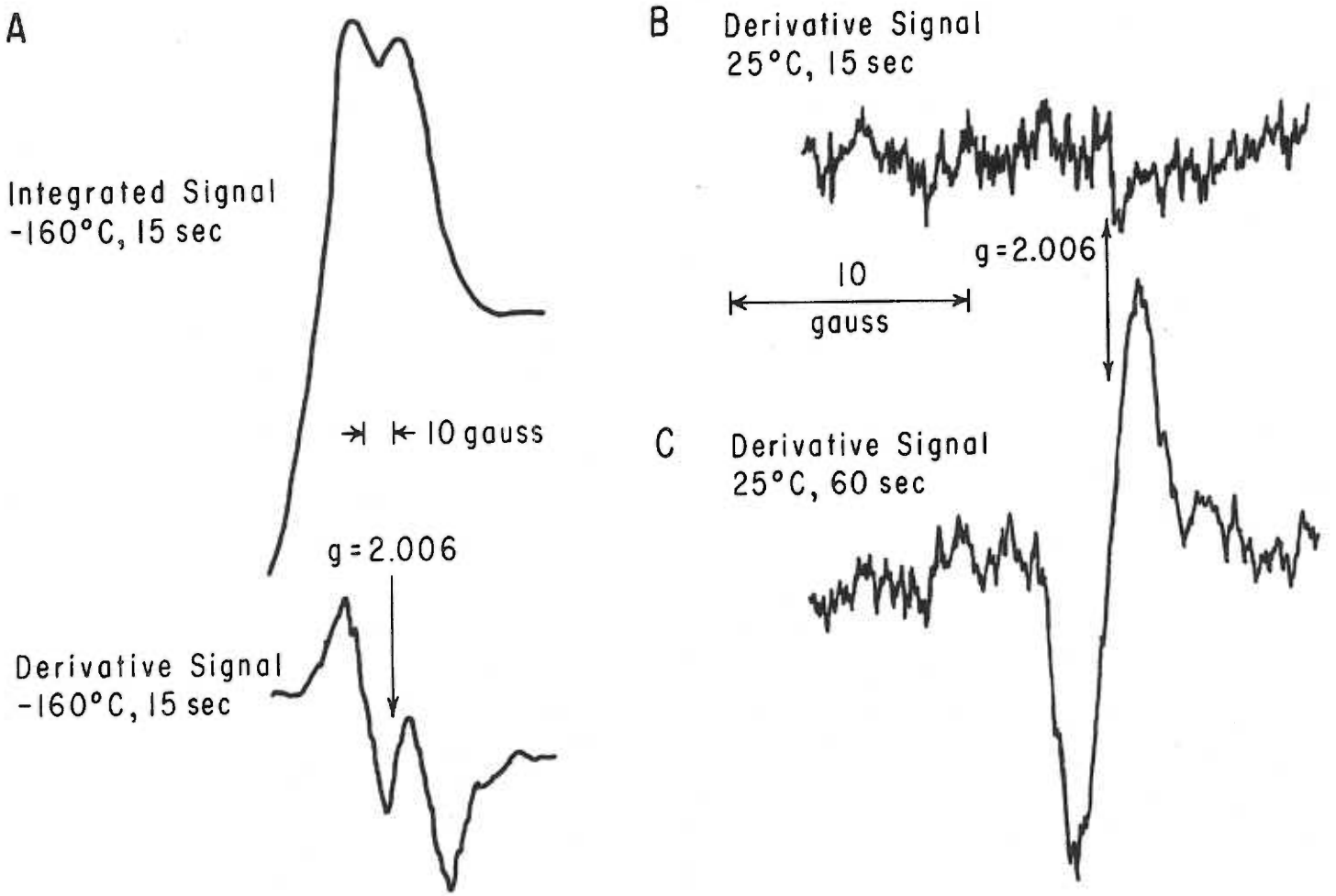

A
Integrated Signal
-160°C, 15 sec
10 gauss
g=2.006
Derivative Signal
-160°C, 15 sec
B
Derivative Signal
25°C, 15 sec
g=2.006
10
gauss
C
Derivative Signal
25°C, 60 sec

FIG. XXIII

RADICAL INTERMEDIATE IN THE OXIDATION OF
4-HYDROXYPHENYL-1-PHOSPHATE

Recorded is the ESR derivative signal obtained from the steady-state radical formed 165 msec. after mixing equivalent volumes of 0.006 M $KMnO_4$ and 0.01 M 4-hydroxyphenyl-1-phosphate at 25°C. in pH 11.7 phosphate buffer. Also recorded is the graphically derived ESR absorption spectrum. This spectrum is composed of 6 lines with a g value of 2.005.

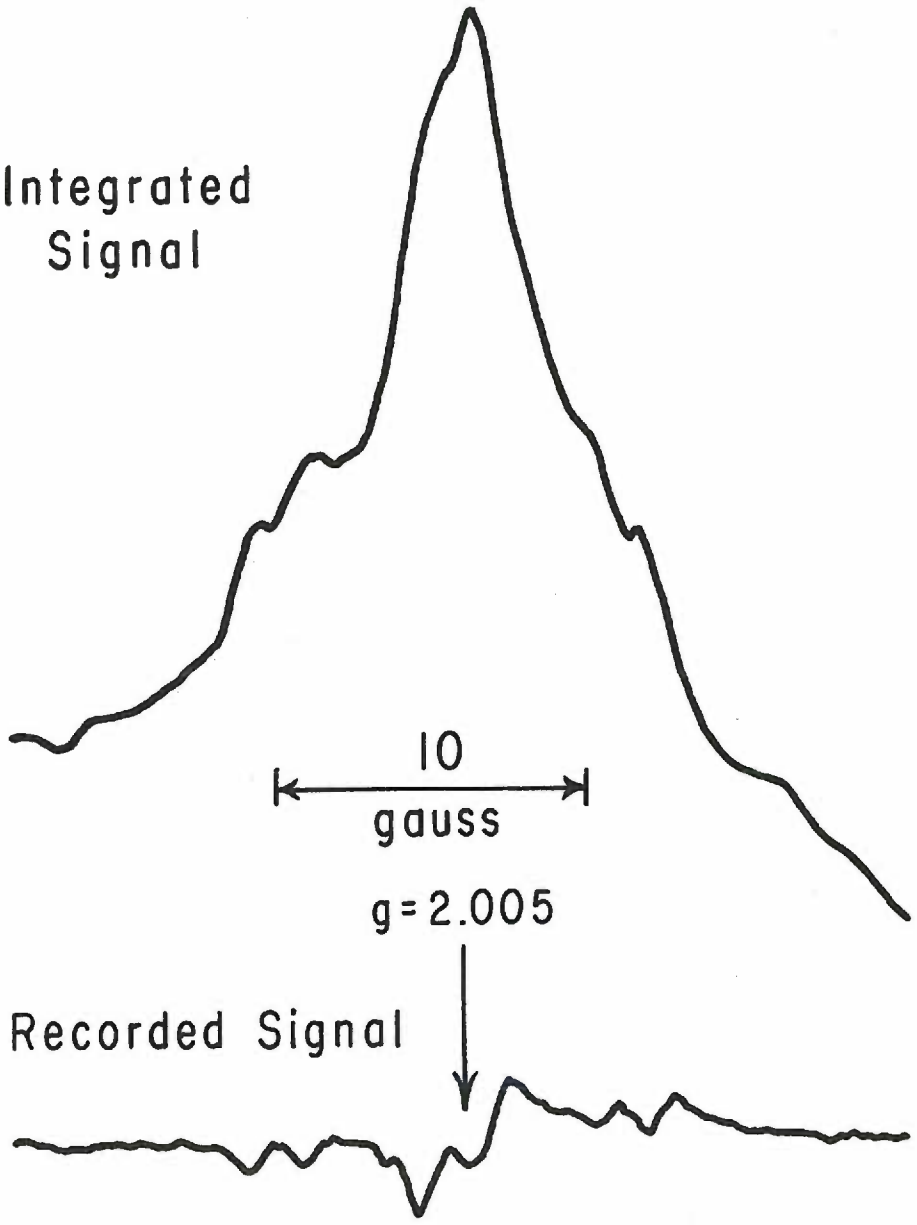

Integrated
Signal
10
gauss
g=2.005
Recorded Signal

FIG. XXIV

RADICAL INTERMEDIATE IN THE OXIDATION OF
4-HYDROXYNAPHTHYL-1-PHOSPHATE

Recorded here is the ESR derivative signal obtained from the steady-state concentration of radical formed at 25°C. when a solution of 0.005 M 4-hydroxynaphthyl-1-phosphate and 0.003 M $KMnO_4$ in 0.55 M Na_2CO_3 is examined 165 msec after mixing. In addition, a graphically derived ESR absorption spectrum is presented. The g value is 2.004. The signal appears to be composed of 10 major lines.

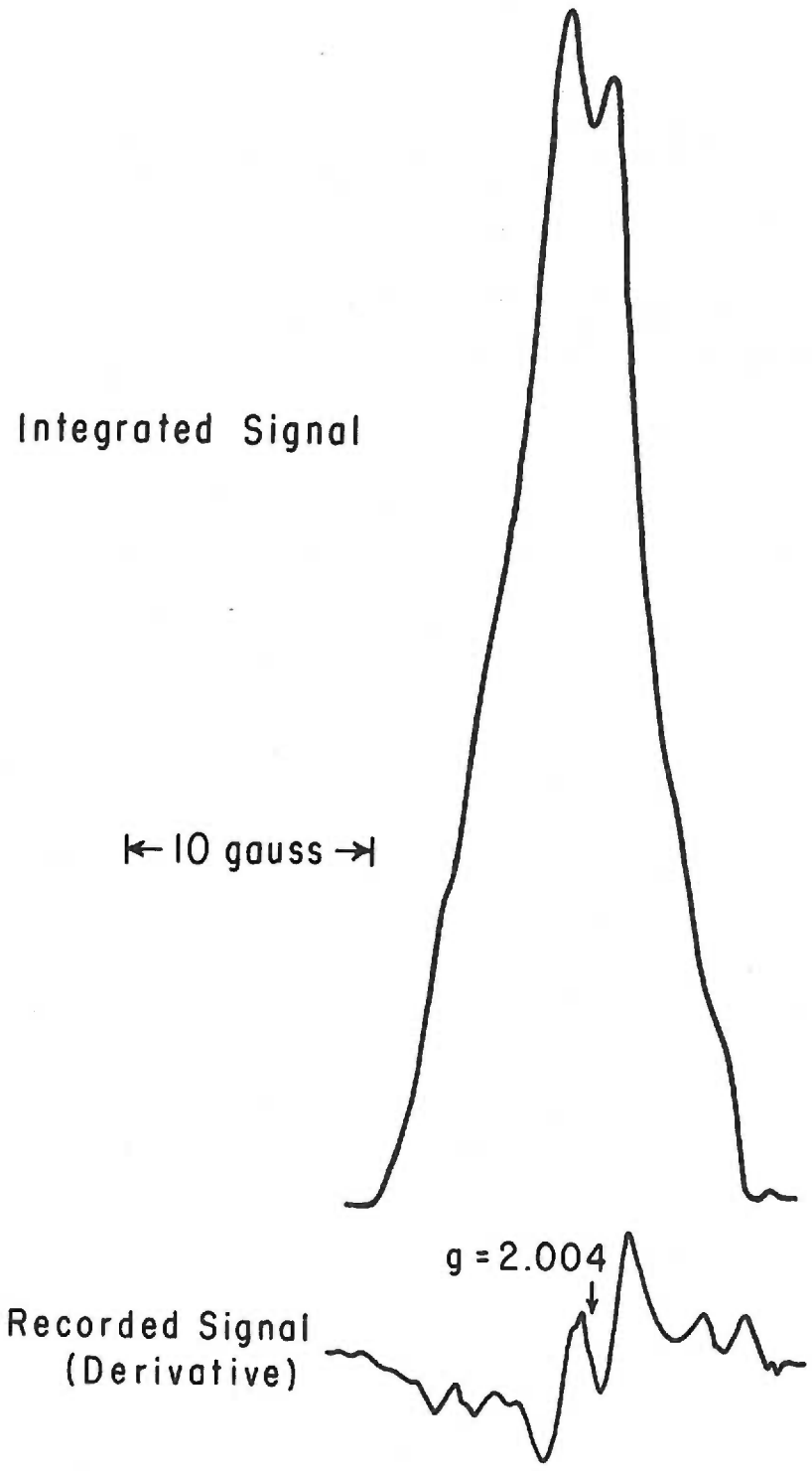

Integrated Signal
10 gauss
g = 2.004
Recorded Signal
(Derivative)

FIG. XXV

RADICAL FORMED BY ULTRAVIOLET IRRADIATION OF A FROZEN 4-HYDROXYPHENYL-1-PHOSPHATE SOLUTION

Recorded is the ESR derivative signal obtained from radicals trapped in ice at -160°C. Radicals were prepared by freezing a 0.01 M solution of 4-hydroxyphenyl-1-phosphate and irradiating with an ultraviolet light source for 90 minutes. A doublet signal of approximately 5 gauss line separation is obtained.

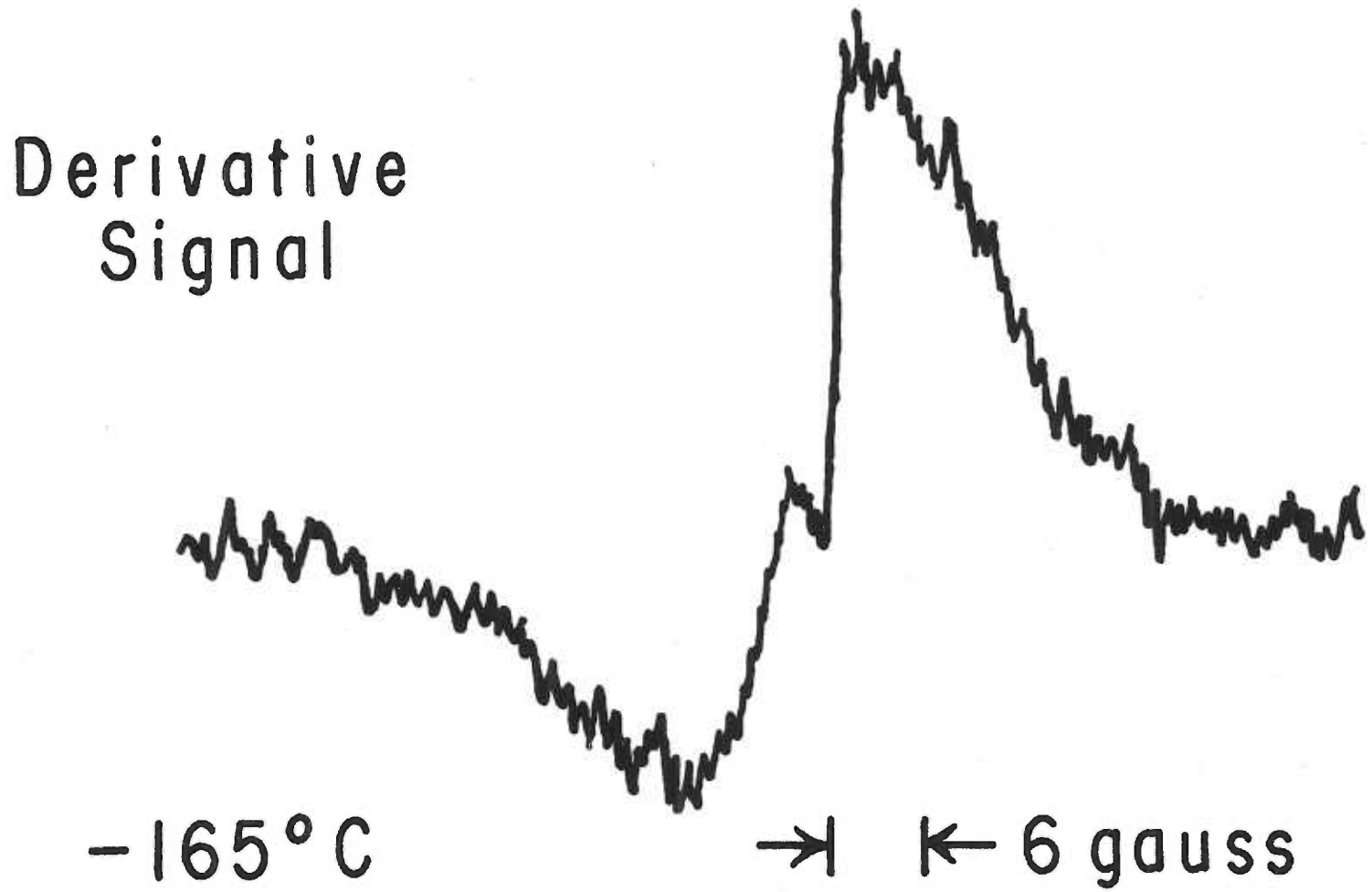
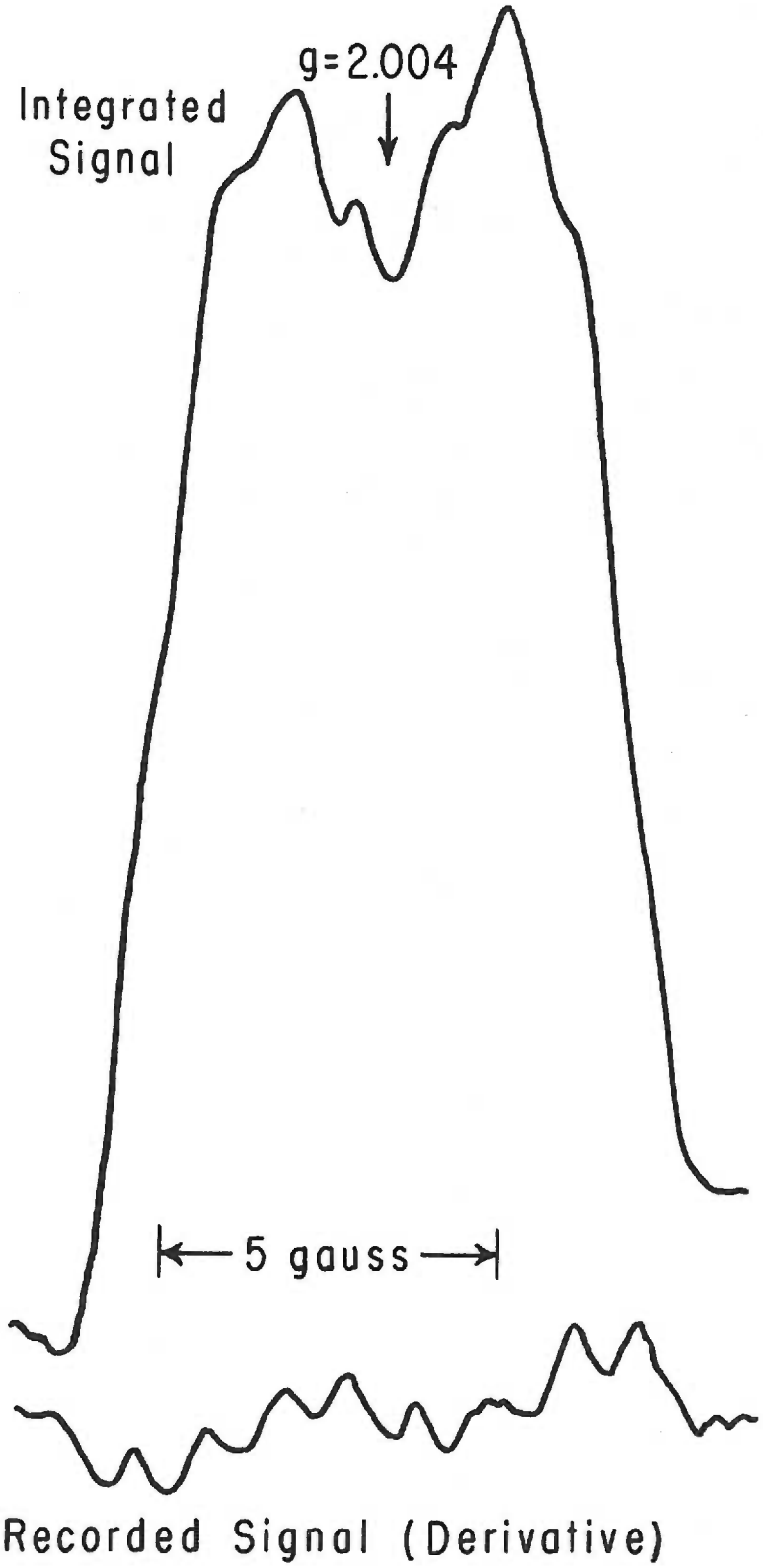

Derivative
Signal
-165°C
6 gauss

FIG. XXVI

RADICAL INTERMEDIATE IN THE OXIDATION OF
2-HYDROXYPHENYL-1-PHOSPHATE

Recorded is the derivative signal and graphically derived ESR absorption spectrum obtained from the steady-state radical produced in a solution of 0.005 M 2-hydroxyphenyl-1-phosphate and 0.003 M $KMnO_4$ in 0.55 M Na_2CO_3 165 msec after mixing. An 8 line spectrum is observed with a g value of 2.004.


g=2.004
Integrated Signal
5 gauss
Recorded Signal (Derivative)

IV. DISCUSSION

The rapid conversion of 4-hydroxy-2,3-dimethylnaphthyl-1-phosphate to 2,3-dimethylnaphthoquinone by peroxidase-H_2O_2 is clearly demonstrated in Fig. XIII. Since peroxidase operates by a free radical mechanism exclusively (28), the 4μM sec.$^{-1}$ change in substrate concentration represents a corresponding increase in radical concentration. Small differences between rates of substrate utilization and product formation, shown in Fig. XIV, are not considered significant. Product extinction did not appear to be constant; consequently reproducibility in these experiments was not good. Presumably, supersaturated solutions of product were formed which did not obey Beer's law.

Failure to detect a radical species spectrophotometrically and the detection of only very low concentrations by ESR spectroscopy, indicate that the radical intermediate has a very short half-life in acid solution. At higher pH, the enzyme oxidation rate decreases more than radical half-life increases and, as before, no radical is detected. Obviously oxidation intermediates decay very rapidly and phosphate transfer progresses more rapidly than can be measured. Therefore, it is not surprising that no detectable differences in oxidation

rate are observed in the presence of divalent cations since their function would be the catalysis of phosphate transfer (86).

Almost quantitative recovery of the quinonoid product indicates that side reactions are unimportant and the reaction proceeds by one mechanism exclusively. Detection of even small radical concentrations therefore, means that that radical is part of the reaction pathway.

Stabilization of short-lived radicals by trapping on various types of ion-exchange or adsorption columns has been observed (10). Increased intensity of ESR signals from an oxidation occurring on TEAE-cellulose columns indicates a stabilization of radical oxidation intermediates. This technique was abandoned in these experiments when it was discovered that the reaction product had been modified.

Integrity of reaction product is of special importance when identification of intermediates is used to determine a specific reaction mechanism. Spectra from side-reaction intermediates would be misleading. In addition, condensation reactions may lead to stable radical species which can be confused with reaction

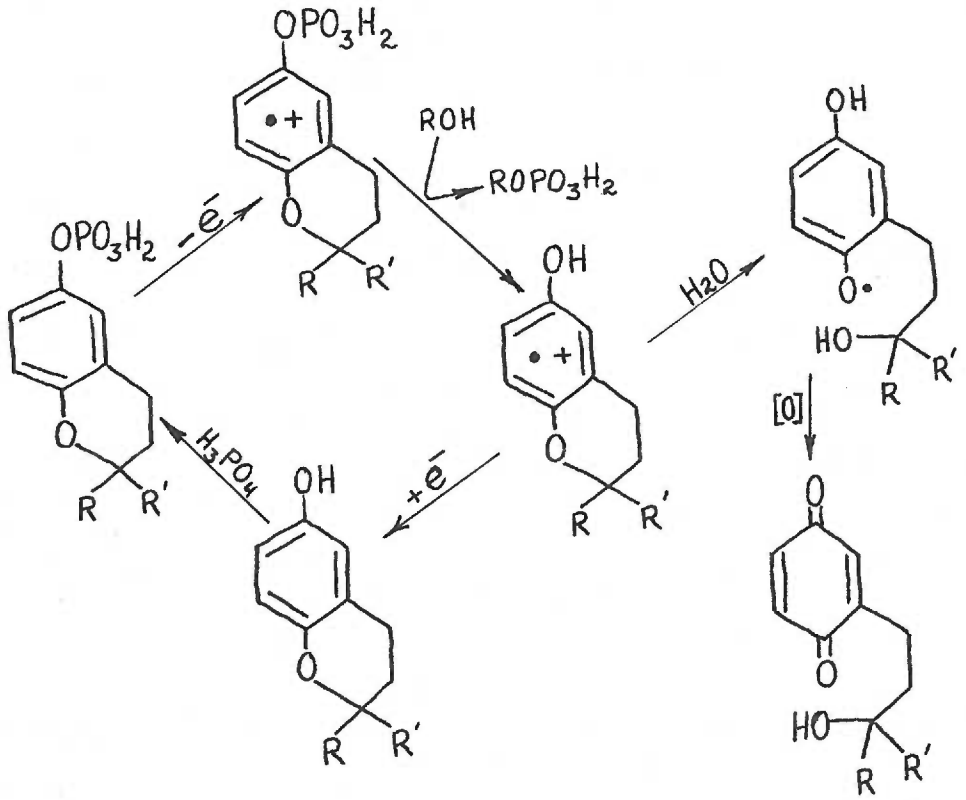
intermediates. For example, a stable radical is observed when 2-hydroxyphenyl-1-phosphate is oxidized by peroxidase-H_2O_2. It has been reported that ortho quinones, the product of this reaction, condense to melanins which form stable radicals (92). The darkened solution observed in the above oxidation indicates a stable melanin radical is formed as a reaction by-product.

Of a variety of strong oxidizing agents chosen for quinol phosphate oxidation, all were observed to produce a free radical intermediate if the proper conditions for detection were satisfied. However, an understanding of reaction mechanism rests on a positive identification of this intermediate. Furthermore, the kinetics of its formation and decay as well as its chemical properties should be determined. This may be done only by generating substantial concentrations of radical under conditions favoring detection of isotropic hyperfine structure.

An instantaneous reaction of quinol phosphate and acid ceric sulfate, known to be a powerful one-electron oxidant, would produce a high initial radical concentration. Experimental results indicate that this ceric sulfate oxidation is relatively slow and that a minimum free radical concentration is produced. On the other hand, chromanyl phosphates appear to be oxidized at

approximately the same rate but rather large free radical concentrations are detected (Figs. XV and XVI).

Formation of high radical concentrations from chromanyl phosphates probably results from slower decay rates in the acid medium. Conceivably, the chromanyl phosphate free radical, initially formed, slowly transfers its phosphate group leaving a chromanoxy free radical. Rapid hydrolysis in acid solution opens the chroman ring to yield a semiquinone. Rapid oxidation and dismutation convert this to quinone under these experimental conditions.



It can be seen that oxidation of chromanyl phosphate by one electron during respiration could lead to phosphate transfer without opening the chroman ring. Compound (C) could be reduced to (F) and be rephosphorylated to (A), the starting product, without producing a quinone. The observation by Boyer (15), that tocopherol may be oxidized in neutral solution to a "tocopheroxide" in which the chroman ring has not been cleaved supports this proposal. Michaelis demonstrated that both the chromanoxy free radical and semiquinone of tocopherol could be formed during oxidation (98). He also pointed out that this chromanoxy free radical could be reduced again without the chroman ring opening. Evidence of quinone function in electron transport is sought by observing changes in its characteristic spectrum during respiration. Failure to observe significant changes in quinone optical density could be the result of cyclic one-electron oxidation-reductions where quinones play no part.

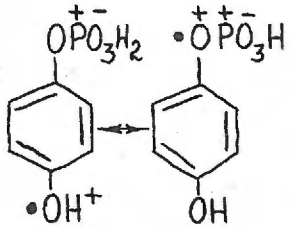
The ESR signals observed from α-tocopheryl phosphate and the 6-chromanyl phosphate of vitamin K_1 are of the same general form. A very broad doublet containing no hyperfine structure is obtained. The broad doublet, no doubt, is related to phosphorus

interaction in a molecule whose positions ortho to the phosphate group have bulky substituents. This will be discussed more fully for 2,3,5,6-tetramethylbenzo-1,4-semiquinone-1-phosphate where a complete interpretation can be made.

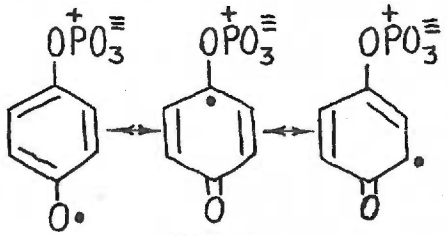
The lack of hyperfine structure in the chromanyl phosphate free radical spectrum results from a dominant anisotropic interaction. These substances are very slightly soluble and must be dispersed as an emulsion. The molecules in the emulsion droplets do not tumble rapidly enough for anisotropic interactions to average out. By dissolving α-tocopheryl quinone in an alcoholic medium, in which it is soluble, adding base and partially reducing the mixture, an ESR spectrum is obtained having abundant hyperfine structure (Fig. XVII). A seventeen line spectrum is observed with a broad, less intense absorption near the extremities of the signal. This signal probably is composed of twenty one equivalently spaced lines. Nine equivalent protons of the methyl groups produce a ten line spectrum which is split into triplets by two methylene protons. The splitting due to the methylene protons is just half that due to methyl protons and a twenty one line spectrum results. Similar results have been reported for coenzyme Q_{10}

semiquinone and vitamin K_1 semiquinone (12).

Semiquinone phosphates should be slightly more stable in alkaline solution than in acid for two reasons. First, acid dissociation of phosphate in alkaline solution leaves the phosphate group with an excess negative charge which tends to neutralize the positive charge on phosphorus. Also, the semiquinone is unprotonated in alkaline solution, thereby maintaining a lower positive character on the bridge oxygen. Both factors reduce static repulsion between the bridge oxygen and phosphorus resulting in a more stable molecule.



Acid



Base

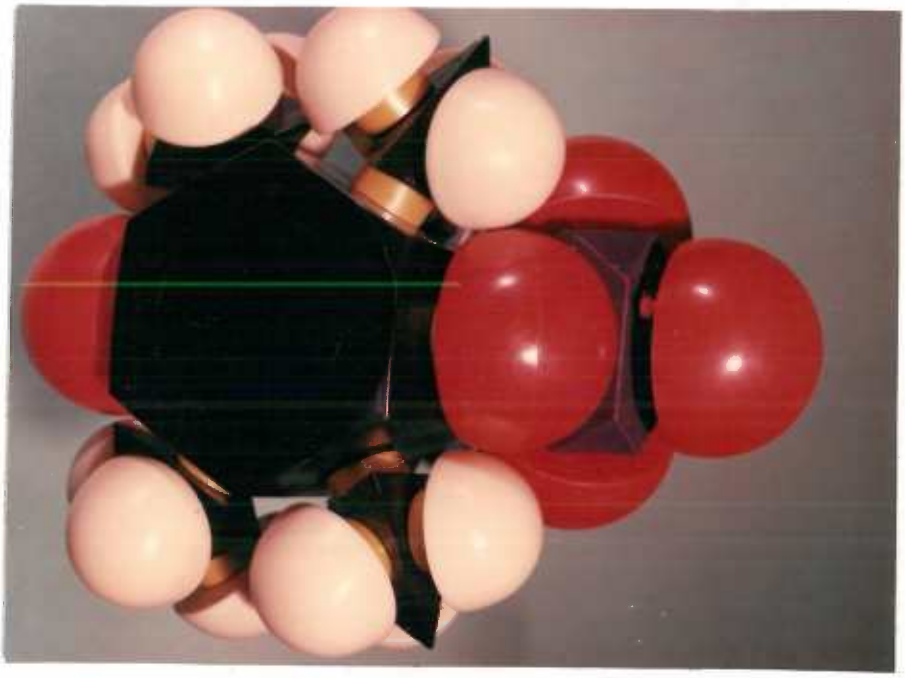
Hypobromite and hypochlorite readily oxidized quinol phosphates but only very small free radical concentrations were produced. Preliminary experiments indicated that other strong oxidizing agents, such as iodate or alkaline ferricyanide, produced useful concentrations of radical from certain quinol phosphates. Alkaline permanganate appeared to oxidize all quinol phosphates with the production of high radical concentrations.

Furthermore, this reagent did not attack the quinone product in alkaline solution.

Examination of ESR spectra from oxidatively produced semiquinone phosphate free radicals reveals a doublet splitting as a common characteristic for all. From the spectrum of 2,3,5,6-tetramethylbenzo-1,4-semiquinone-1-phosphate shown in Fig. XVIII, it may be perceived that the 10 major absorption lines are composed of two overlapping septets. The intensity ratios of these 10 lines is 1:6:15.7:20.4:19.5:19.5:20.4:15.8:6:1, which is in good agreement with predicted intensity ratios for two overlapping septets. The magnitude of the splittings from both the primary doublet and secondary septet is much greater than has been reported previously for semiquinone derivatives (1). An explanation of these results ascribes the primary doublet to an interaction between the unpaired electron and the phosphorus nucleus of spin $\frac{1}{2}$. This must modify the usual semiquinone electron distribution so that abnormally high splittings are obtained from some methyl protons.

Since the phosphorus splitting constant is high while its nuclear magnetic moment is small, interaction must differ from that usually encountered in semiquinone derivatives. It is proposed that bulky ortho substi-

tuents hinder phosphate rotation and cause the phosphate group to be fixed perpendicular to the plane of the aromatic ring. In this position, the lone pair of p electrons on the bridge oxygen are not in a position to conjugate with the aromatic π molecular orbital.

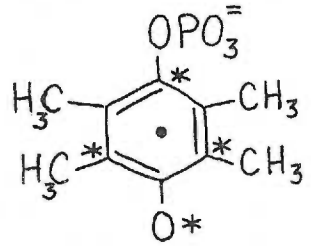


Since the bridge oxygen is unconjugated, this molecule becomes electronically equivalent to a phenoxy free radical. Electron distribution in such molecules approaches that in an alternate hydrocarbon. Becconsall, Clough and Scott (7) measured splittings from numerous phenoxy free radicals and observed a high electron density on the position para to the oxy group. This is

the position occupied by phosphate in semiquinone phosphates.

Molecular models show that the phosphorus nucleus is in very close proximity to this carbon. Overlap of the π molecular orbital, in which resides the unpaired electron, with an s orbital of phosphorus would form a weak bond. This new hybrid orbital would place the unpaired electron in direct contact with the phosphorus nucleus and a strong isotropic hyperfine interaction would occur. The strength of the interaction would be proportional to electron density on the carbon to which the phosphate group is attached.

The "alternate hydrocarbon" structure for durosemiquinone phosphate is shown below. Carbons marked with asterisks have high unpaired electron density and



those without markings have little or no unpaired electron density associated with them. From this diagram, it can be seen that two methyl groups have high unpaired electron

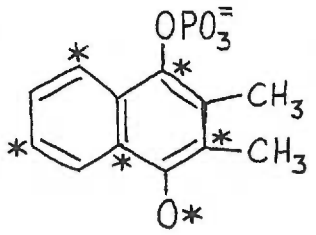
density and consequently, a higher splitting constant than usually obtained from semiquinone derivatives should be observed. The secondary septet of the durosemiquinone phosphate spectrum is derived from six equivalent methyl protons at these positions, and the magnitude of the splitting constant, 5.4 gauss, is in accord with that reported for such positions in phenoxy free radicals.

The observed signal contains additional hyperfine structure derived from the six equivalent methyl protons at positions having no unpaired electron density. Theory predicts no interaction may occur in the absence of unpaired spin density but while unpaired electron density is calculated to be zero, unpaired spin density may assume an absolute value. That is, electron density takes only positive values but spin density may assume values of either sign which is not necessarily proportional to the electron density. The detected interaction appears the same whether caused by positive or negative spin density. In this way, the interactions at positions of no unpaired electron density may be explained as due to interaction with a negative spin density.

Negative spin densities result when a high unpaired

electron density on one carbon induces a spin on a neighboring carbon. The total spin on the molecule must add up to one and the higher spin density on carbons having high unpaired electron density must be neutralized by higher negative spin densities on alternate carbons. For durosemiquinone phosphate, this negative spin density is low; consequently the tertiary septet has a low splitting constant. An interpretation of the 2,3,5,6-tetramethylbenzo-1,4-semiquinone-1-phosphate spectrum is depicted in Fig.XXVII.

The spectrum of 2,3-dimethylnaphtho-1,4-semiquinone-1-phosphate shown in Fig.XIX is in complete agreement with the above interpretation. A six line spectrum with the intensity ratio of 1:3:4:4:3:1 is obtained. This is obviously composed of two overlapping four line spectra. Positions ortho to the phosphate are substituted and the line separation of the primary doublet is 18.5 gauss. Since the oxygen bridge is not conjugated, the following "alternate hydrocarbon" unpaired electron distribution is observed, where positions of high density are marked with an asterisk.



The secondary quartet splitting is due to interaction with three equivalent methyl protons at position-3-, which bears an asterisk in the above figure. As expected for a substance with phenoxy free radical character, the magnitude of this splitting is very large. The remaining hyperfine structure appears to result from each quartet being split into a quartet and a triplet consecutively. This results from three equivalent methyl protons in the -2- position producing a minor quartet and two equivalent ring protons at positions -6- and -8- producing the final triplet.

Phenoxy free radical character also is demonstrated by the magnitude of splitting in the -6- and -8- positions. This is approximately twice as great as is usually observed from semiquinone derivatives (1). Despite this increase in splitting constant at these positions, higher electron density in the oxygen-binding ring allows the negative spin density interaction at position -2- to

dominate. No doubt, negative spin density interactions occur at positions -5- and -7- but the magnitude of splitting is too small to detect. An interpretation of this spectrum is depicted in Fig.XXVIII.

The spectrum of 2-methylnaphtho-1,4-semiquinone-1-phosphate, shown in Fig. XX, also demonstrates the effect of ortho substituents. A primary doublet splitting of 18 gauss is especially obvious. Nonsynchronous overlap of the various signal components obscures most hyperfine structure but an interpretation, depicted in Fig. XXIX, is in agreement with the predicted spectrum. The observed broad triplet is composed of a 3.1 gauss doublet, a 2.3 gauss quartet and a 1.3 gauss triplet. These splittings are due respectively to a proton in the -3- position, three methyl protons in the -2- position and two ring protons in the -6- and -8- positions.

The spectrum of 3-chloro-2-methylnaphtho-1,4-semiquinone-1-phosphate, shown in Fig. XXI, is difficult to interpret because of the presence of a signal from a contaminating radical species. However, the primary doublet of 20.2 gauss splitting due to phosphorus interaction is quite obvious.

Tetrachlorobenzo-1,4-semiquinone-1-phosphate produces a doublet spectrum only, as is shown in Fig XXII.

Strong phosphorus interaction would be predicted since positions ortho to the phosphate are substituted with bulky groups. Chlorine possesses a spin of 3/2 but its nuclear magnetic moment is very small. Consequently, no hyperfine interaction of a detectable magnitude should occur with any of the chlorine nuclei (121).

The concentration of tetrachlorobenzosemiquinone phosphate was low and while it was easily detected at low temperature, its characteristic doublet could not be observed at room temperature. Repeated searching for this signal at increasing periods of time after mixing the reagents, revealed a sharp singlet signal appearing about forty seconds after the reagents were mixed. This signal rose to a maximum in two minutes and decayed slowly with an approximate half-life of three minutes.

The spectrum of this new radical species contained only a singlet absorption and no hyperfine structure. Its g value was 2.006. This spectrum fit the literature description of 2,3,5,6-tetrachlorobenzo-1,4-semiquinone anion (121). In the presence of excess oxidizing agent, there is but one way to form this semiquinone from semiquinone phosphate. A transfer of the phosphate from semiquinone phosphate must occur without a change in oxidation state. Semiquinone is detected in this

instance because the redox potential is sufficiently great to allow detectable concentrations to accumulate, even in the presence of oxidizing agent.

Semiquinone phosphates on which positions ortho to the phosphate are unsubstituted, yield spectra very different from those whose ortho positions are substituted. Free rotation of the phosphate group allows the bridge oxygen to conjugate with the aromatic system and electron distribution approaches that found in semiquinone derivatives. However, static repulsion of electrons by the highly negative phosphate group tends to reduce electron density on the phosphate end of the molecule while increasing it at the other. Consequently, a larger than usual splitting constant for ring proton interactions can be expected. Phosphorus interaction should be reduced to a minimum and ring proton interactions should be essentially equivalent.

The spectrum of benzo-1,4-semiquinone-1-phosphate, shown in Fig. XXIII, contains no indication of a large primary doublet. The interpretation depicted in Fig. XXX, shows this signal may be reproduced by two consecutive triplets split into a minor doublet. The larger triplet is derived from two equivalent protons in the -3- and -5- positions and the smaller triplet is derived from equiva-

lent protons in the -2- and -6- positions. The 1.8 gauss doublet is attributed to a residual phosphorus interaction.

Naphtho-1,4-semiquinone-1-phosphate produces a spectrum which again contains a minor 1.8 gauss doublet (Fig. XXIV). The interpretation depicted in Fig. XXXI shows the protons in the -3- and -2- positions are nearly equivalent, producing consecutive doublet splittings of 5 and 4.5 gauss, respectively. These are split by a small doublet from phosphorus interaction which is split further into a quintet by four equivalent protons in the adjacent ring.

The phosphate group of 4-hydroxyphenyl-1-phosphate extends from the plane of the aromatic ring and consequently, the bridge oxygen is unconjugated*. When this substance was converted to a semiquinone phosphate by U.V. irradiation of its frozen solution, the spectrum obtained at -160°C contained a more marked doublet splitting than was observed in its room temperature spectrum. The low temperature spectrum is shown in Fig. XXV. The increased splitting of the phosphorus indicates the rotational freedom of the phosphate group is restricted in the rigid ice matrix. The molecule is

* W. T. Simpson, Personal Communication. 1963.

frozen while the bridge oxygen is in an unconjugated condition; consequently it remains unconjugated in the semiquinone phosphate. Phosphorus interaction is stronger, therefore, and a greater line separation is observed.

In Fig. XXVI is the spectrum of benzo-1,2-semiquinone-1-phosphate. This very narrow signal contains abundant hyperfine structure but an interpretation is not possible at this time.

From these spectra, it is apparent that semiquinone phosphates and chromanyl phosphates on which positions ortho to the phosphate group are substituted, have electron distributions and chemical properties characteristic of phenoxy free radicals. When positions ortho to the phosphate group are unsubstituted, the semiquinone phosphates are more nearly related to semiquinones. A quantitative measure of pyrophosphate formed by their decay was not made so the phosphorylating properties of these different compounds remains unknown.

Electrolytic oxidation is a very desirable way to form semiquinone phosphate for study of the decay

kinetics (105). Such reaction variables as ionic strength, pH, and temperature may be changed without greatly effecting the mechanism of radical production. In addition, oxidation is stopped immediately when current is switched off and a decay curve would represent only the disappearance of radical species. Unfortunately, the electrolytic cells available commercially are inefficient and a reasonable current can not be made to flow without increasing voltages to unreasonable values. At higher voltages, many undesirable reactions occur (2) and kinetic measurements are meaningless.

The failure of attempts to show radical signals, characteristic of semiquinone phosphates, were associated with actively phosphorylating mitochondria or M. phlei fractions does not mean these radicals are absent from the living systems. It means only that a sufficiently high concentration of them to allow identification is not present. Such a result would be expected when the short half-lives of these radicals and the slow oxidation rate of biological systems is considered.

Peroxidase and H_2O_2, at higher than physiological concentrations, produces only small concentrations of radical, yet large quantities of substrate are oxidized within short periods of time by a free radical mechanism.

Peroxidase also oxidizes the 6- chromanyl phosphate of vitamin K_1 with the production of small but significant concentrations of a radical intermediate. This chromanyl phosphate reportedly transfers its phosphate group to adenosine diphosphate during oxidative phosphorylation in M. phlei fractions (4).

Observation of direct phosphate transfer from the hydroquinone monophosphate of coenzyme Q to ADP during oxidative phosphorylation in rat-heart mitochondria indicates that semiquinone phosphates are involved in the primary act of energy conservation in mammals.

It is inferred that the formation of a chromanyl phosphate free radical represents an energy trapping mechanism of oxidative phosphorylation in M. phlei and formation of semiquinone phosphate serves the same purpose in mammals. These organisms are located at the extremes of the phylogenetic scale. Therefore, it is probable that interlocated respiring organisms utilize this same oxidative phosphorylation mechanism.

Fig. XXVII

2,3,5,6-Tetramethylbenzo-1,4-semiquinone-1-phosphate

FIG. XXVIII

The interpretation of the spectrum of 2,3-dimethyl-naphtho-1,4-semiquinone-1-phosphate depicted here is modified slightly from that reported earlier for this compound (13).

2, 3 - dimethylnaphtho - 1,4 - semiquinone - 1 - phosphate

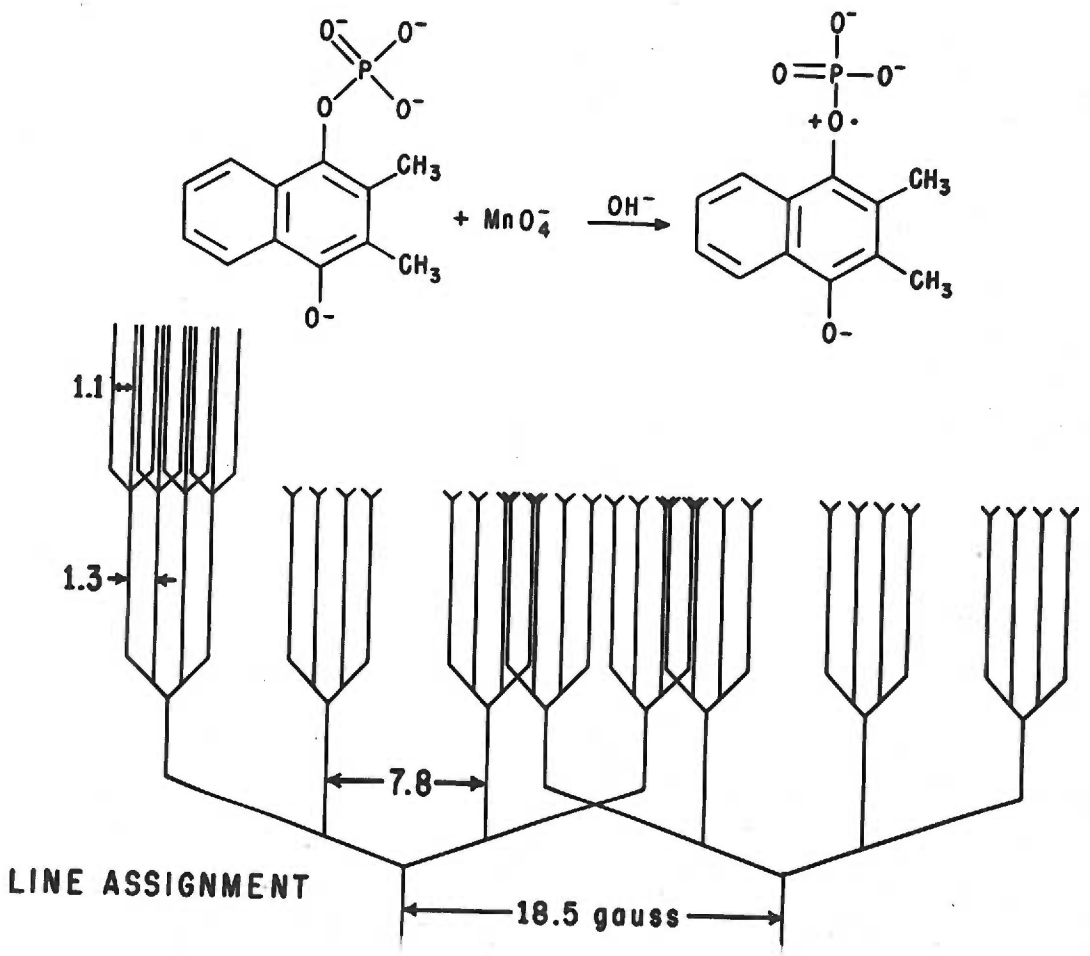
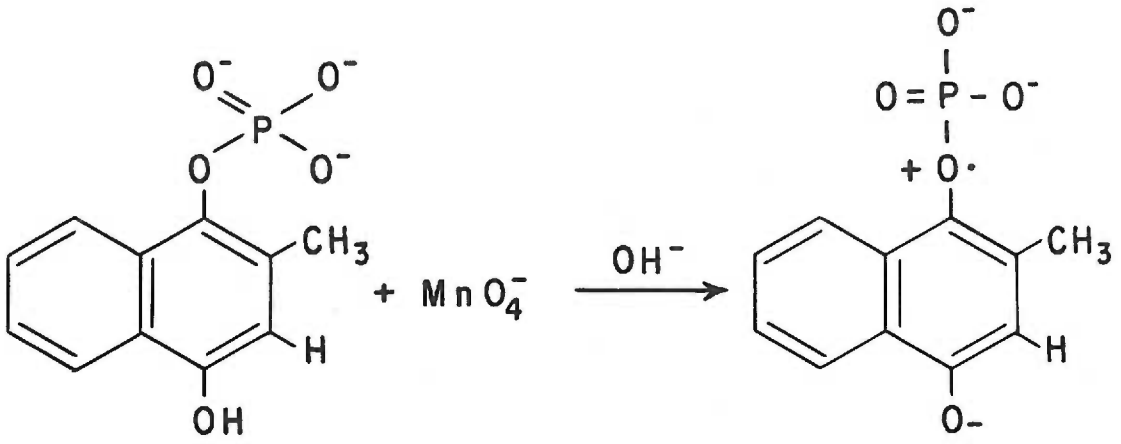


Fig. XXIX

2-methylnaphtho-1,4-semiquinone-1-phosphate



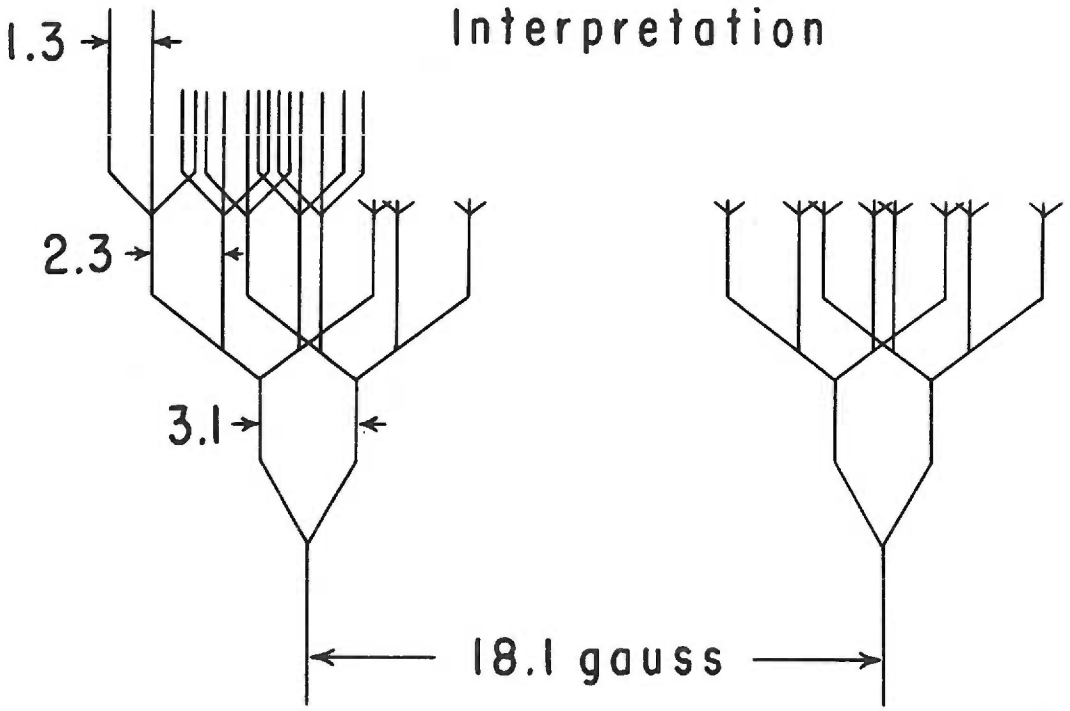
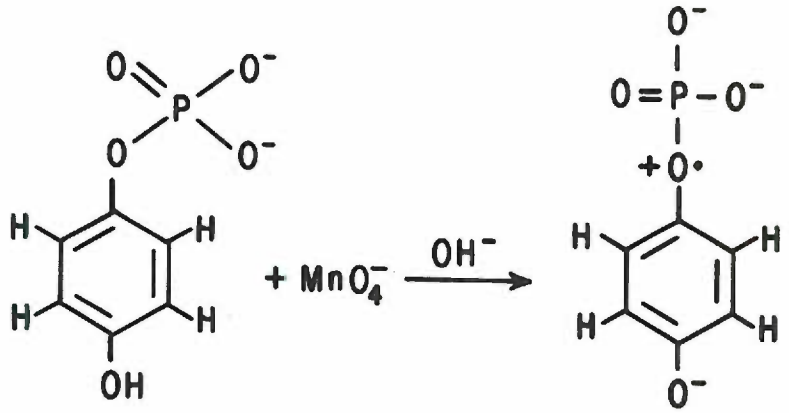


Fig.XXX

Benzo-1,4-semiquinone-1-phosphate



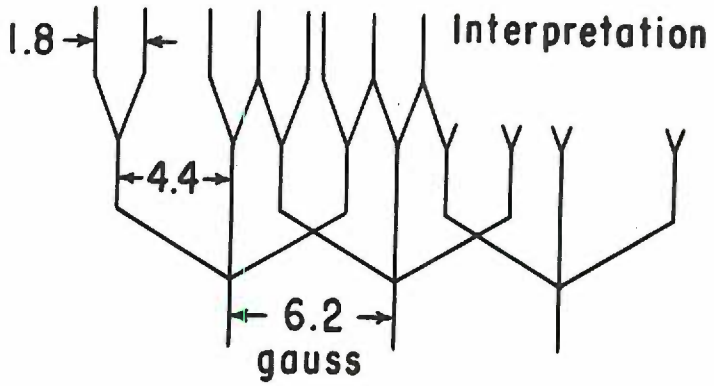
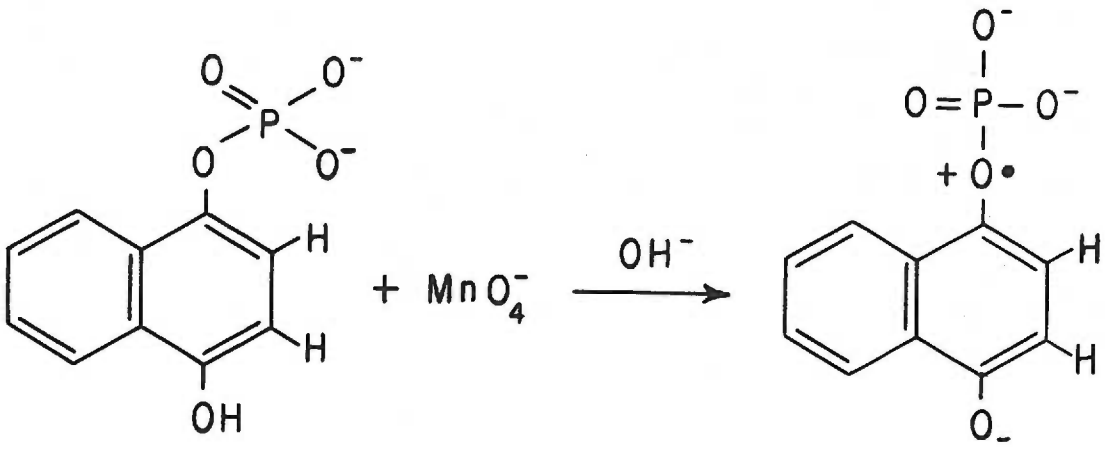


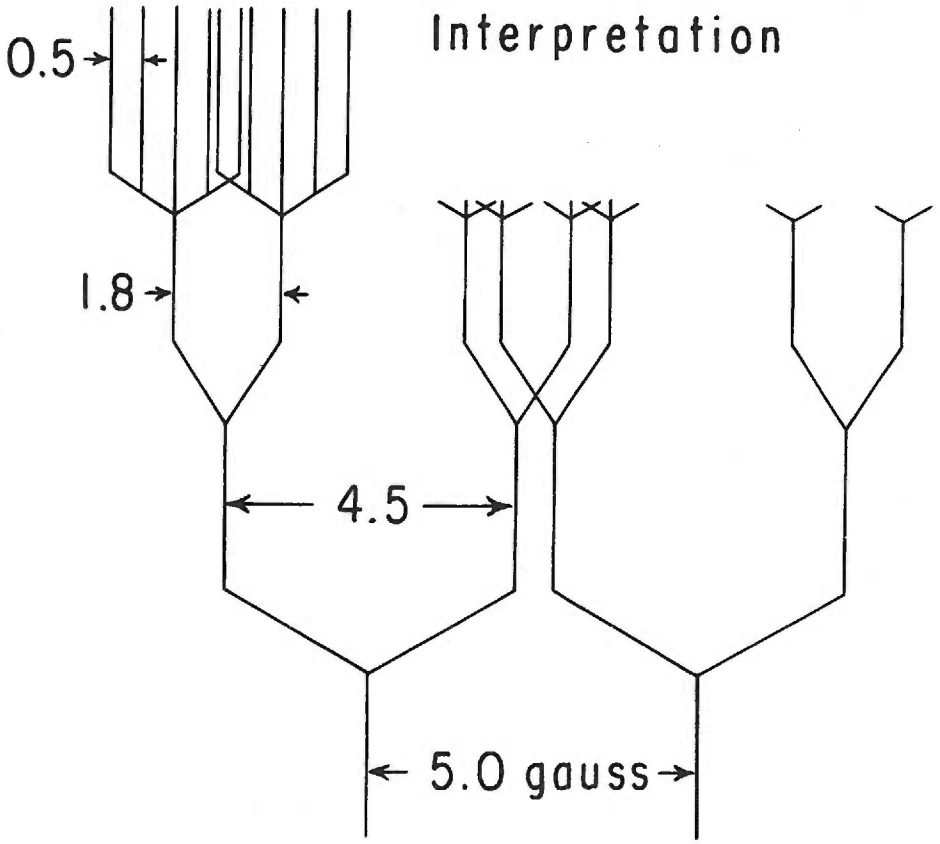
FIG. XXXI

NAPHTHO-1,4-SEMIQUINONE-1-PHOSPHATE

Lack of hyperfine structure makes a conclusive interpretation impossible. However, this interpretation agrees with the recorded signal and the magnitude of splitting is in agreement with that observed from benzo-1,4-semiquinone-1-phosphate.

Naphtho-1,4-semiquinone-1-phosphate





Interpretation

From these experimental results, it is concluded that quinol phosphates are converted to quinones and high transfer potential phosphate compounds by two one-electron oxidation steps. The free radical intermediate, semiquinone phosphate, transfers its phosphate group without further change in oxidation state. Both pyrophosphate and semiquinone are products of the transfer reaction. The same mechanism of oxidation and phosphorylation appears to function with 6-chromanyl phosphates. Both semiquinone phosphates and 6-chromanyl phosphate free radicals are active phosphorylating agents.

The mechanism of phosphate transfer is still uncertain. Semiquinones and chromanoxy free radicals dismutate or undergo further oxidation to quinones under the conditions of these experiments. However, in biological systems, they may be reduced to quinols without quinone formation.

These results support the premise that oxidation of a 6-chromanyl phosphate by M. phlei fractions constitutes the energy trapping mechanism of oxidative phosphorylation in that organism and that oxidation of quinol phosphate serves the same function in mammals.

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