DETERMINATION OF A FREE RADICAL FORMATION

CONSTANT; AND A FREE RADICAL SURVEY OF

SOME FOODSTUFFS

by

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A THESIS

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I INTRODUCTION

A. OBJECTIVES

The work herein described involves quantitative application of election paramagnetic resonance spectroscopy in two investigations: 1, Determination of the free radical formation constant of an electron transfer oxidase, lactic oxidative decarboxylase, and 2, A survey of some foodstuffs for their free radical content.

In keeping with Lavoisier's definition, "All science is formed necessarily of three things: the series of facts which constitute the science; the ideas which they call forth; the words which express them", it seems appropriate here, to review at least briefly the development of current concepts of oxidation and reduction; and the application of electron paramagnetic resonance spectroscopy to the question of the biological occurrence of free radicals.

B. HISTORICAL

The problems of combustion and chemical oxidation were naturally of great importance throughout the long development of the science of chemistry. Around the beginning of the eighteenth century, Becher and Stahl developed the phlogiston theory which held sway until 1775, at which time Antoine Laurent Lavoisier demonstrated that a certain constituent of air entered into the

formation of definite compounds. In November 1779, he suggested the name oxygen for this active gas. Although Lavoisier still thought of heat as a material, "caloric", nevertheless this period marked the dawn of modern chemistry, (18).

Lavoisier's work, of course, led to the use of the term oxidation for combustion and for a number of other reactions.

In 1913, G. J. Moseley, who was working with Rutherford at the University of Manchester, discovered the correlation between the frequencies of X-rays and the particular target metal used for their production, and then announced the principle of atomic numbers. This discovery coupled with Niels Bohr's atomic model cleared the way for development of the modern theory of chemical valence by G. N. Lewis, Walther Kossel and Irving Langmuir, through the period from 1916 to 1923, (18).

The new concepts of valence automatically led to a broad definition of oxidation as, the loss of one or more electrons by an atom with a resultant increase in positive charge or valence of the atom or group with which it is associated, and reduction as the converse. It was also easy to understand that each unit of oxidation required a corresponding unit of reduction.

C. CURRENT CONCEPTS OF OXIDATION-REDUCTION

1. Oxidation-Reduction In General

Oxidation-reduction reactions, or to use the more coherent terse term, redox reactions, are among the most fundamental and important events in biology. The broad concept of the term extends from actual transfer of electrons to a mere shift of influence over them, i.e., from ionic to covalent bond formation and disruption. Implicit in this definition, are, at least indirectly, all chemical reactions except hydrolysis and neutralization; thus the formation of all compounds, biologic and geologic; and the major energy transfers and transformations from the radiant energy of the sun through plants and animals, resulting in the ecological panorama, both spatial and temporal, of the altered biota and geology, including the tremendous deposits of coal and hydrocarbons.

a. Early Modern Concepts

The basic cause of redox reactions is found in Kosel's octet theory which was further elaborated by Lewis and Langmuir. According to the octet theory the most stable configuration for an atom is that in which the outermost electron shell or compliment is the same as that of one of the inert gasses, i.e., two electrons for hydrogen corresponding to helium and eight electrons for all other elements corresponding to: Ne, A, Kr, Xe,

and Rn. Thus when sodium and chlorine are brought together; the sodium readily releases its lone electron from the M shell leaving its L shell with a stable number of eight electrons, while chlorine with seven electrons in its outer shell avidly acquires the single electron released by the sodium to bring its M shell up to eight. The sodium atom by losing one electron has been oxidized, acquiring a positive charge of one, while the chlorine atom has, by gaining one electron, been reduced and has a negative charge of one.

$$Na \cdot \ + \ \cdot \ddot{\underset{\cdot\cdot}{Cl}} : \ \longrightarrow \ Na^+ \ + \ \left[: \ddot{\underset{\cdot\cdot}{Cl}} : \right]^-$$

The resultant charged atoms now called ions are held togehter in the solid state by their opposite electric charges, but when dissolved in a polar solvent or when in the molten state they can separate.

The foregoing description applies to the formation of ionic bonds, but the large majority of organic compound bonds, and many (inorganic) compound bonds are the covalent type. The octet rule also applies here but the electrons instead of being completely transferred are merely shared. Thus when a methane molecule is formed four hydrogen atoms each contribute their lone k shell electron and one carbon atom its four L shell electrons, to establish a stable ring of eight electrons about the

carbon atom, but this is also equivalent to four sets
of two electrons each for the four hydrogen atoms.

$$4H + C \longrightarrow H\!:\!\overset{\overset{\displaystyle H}{\cdot\cdot}}{\underset{\overset{\cdot\cdot}{\displaystyle H}}{C}}\!:\!H$$

The same type of mechanism produces the non-ionic part
of inorganic radicals such as SO_4^{--} and ClO_3^{-}.

Covalent bonds are also formed by the coordinate
covalent mechanism in which both electrons of a covalent
bond are provided by one of the atoms e. g. Ammonia
can use its unshared pair of electrons to combine with
a hydrogen ion to form the ammonium radical

$$H\!:\!\overset{\overset{\displaystyle H}{\cdot\cdot}}{\underset{\overset{\cdot\cdot}{\displaystyle H}}{N}}\!: \;+\; H^+ \longrightarrow \left[H\!:\!\overset{\overset{\displaystyle H}{\cdot\cdot}}{\underset{\overset{\cdot\cdot}{\displaystyle H}}{N}}\!:\!H \right]^{+}$$

Following incorporation the hydrogen ion loses its
identity and is indistinguishable from the other hydrogen
atoms.

There is another type of bonding which at first
sight appears to violate the octet rule, viz., in compounds
in which some or all of the bonds are single shared
electrons, e. g.

$$B_2H_6 \quad \text{or} \quad H\!:\!\overset{\overset{\displaystyle H}{\cdot\cdot}}{\underset{\overset{\cdot\cdot}{\displaystyle H}}{B}}\!:\!\overset{\overset{\displaystyle H}{\cdot\cdot}}{\underset{\overset{\cdot\cdot}{\displaystyle H}}{B}}\!:\!H$$
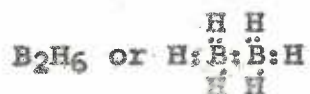
Sugden (20) suggested that this sharing of single
electrons, and the high stability of some such compounds
is explained by the resonance concept of quantum

6.

mechanics, viz., that single electrons oscillate between locations to provide a stable configuration at each location part of the time. This argument also applies to the hydrogen-molecule ion, $(H \cdot H)^+$

b. Recent Modern Concepts

Two theories based on wave mechanics have been advanced to account for valence; (20) the Heitler-London and the Hund-Mulliken.

The Heitler-London theory attributes the valence of an element to the presence in the external shell of an atom of electrons with unpaired spins, +1/2 or -1/2, and these can satisfy their tendancy for spin coupling by combining with electrons of opposite spin on other atoms to form a valence bond. This theory is also referred to as the homopolar method since it is mainly applicable to covalent bond.

The Hund-Mulliken theory or as it is called the molecular orbital theory sets up a wave equation containing both homopolar and ionic binding terms. If the values of the homopolar terms are considerably greater than those of the ionic terms the bonding is essentially covalent, but if the ionic terms predominate the bonding is ionic. Mixed types of binding occur between these two extremes, resulting in molecules with permanent dipole moments. This method segregates the electrons of a molecule into three

categories; bonding, antibonding, and nonbonding; the stability of a molecule being a reflection of the ratio of bonding to antibonding electrons.

With either theory mathematical difficulties have prevented exact treatment of all but the very simple molecules such as the hydrogen molecule and the hydrogen-molecule ion. With more complex systems it has been necessary to use approximations, which have however furnished much information about valence and bonding.

The current concepts of oxidation are, of course, applicable to those cases covered by the old classical definition of oxidation, in which oxygen necessarily participated in the reaction: e.g.

$$2H_2 + O_2 \longrightarrow 2H_2O$$

$$2C_2H_5OH + O_2 \longrightarrow 2CH_3CHO + 2H_2O$$

$$S + O_2 \longrightarrow SO_2$$

$$4Fe + 3 O_2 \longrightarrow 2Fe_2O_3$$

In all redox reactions the oxidant acquires more influence over the electrons involved than does the reductant. In the case of $H:O:H$ dissociation of a hydrogen ion does occur, alcohols release the hydroxyl hydrogen with relative ease, etc.

D. CONDITIONS ESSENTIAL FOR OXIDATION AND REDUCTION.

An equation such as

$$A^+ + B \longrightarrow A + B^+$$

implies several conditions:

a. Electron transfer is possible. Either the reactants are in contact, as in solution, in gas, or in the molten state; or there is a conducting path between the reactants for the transfer of electrons, e.g., a metal conductor, an intermediate redox system, or a charge transfer process, (to be discussed later).

b. Any existing energy barriers have been overcome.

c. There is a transfer of electrons in one direction, i.e., the reaction goes to completion either because A has a much stronger attraction for the reducing electrons than has B or the system is open, that is the products are removed, otherwise equilibrium would occur requiring the double arrow.

1. Redox Potentials.

The effectiveness of a substance as an oxidant or reductant is a reflection of its relative attraction for electrons as compared to that of other substances. oxidising effectiveness (electron pressure) is expressed as a potential in volts, and the standard of comparison is the hydrogen-gas electrode; which consists of a platinized platinum electrode exposed to hydrogen gas at a pressure of one atm and immersed in a solution of hydrogen ions at an effective concentration of one, i.e. activity=1, and assigned zero

potential. As the hydrogen electrode is difficult to construct and maintain it is rarely used directly, instead one such as the calomel electrode which is easily constructed is used, the difference in potential between the two standards being constant. The potential of a substance is determined by using it as a half cell, in a separate container with a platinum electrode, against the standard, a salt bridge connects the two containers and the electrodes are connected through a potentiometer. If the oxidising power of the substance is greater than that of hydrogen the potential is positive, if less it is negative. When the substance is at one molal concentration and the system is at $25^{o}C$, the potential found is called the normal electrode potential. If the comparison half cell contains, not just the fully oxidised form of the substance, but instead a mixture of one-half oxidised and one-half reduced, other conditions being equal the potential found is called the normal redox potential, e.g.

System(pH 7)	$E_o^!$ Standard Redox Potential. Volts
$\frac{1}{2}O_2 : H_2O$	+0.82
O_2 (1 atm.) : OH^- (pH 7)	+0.810
$Fe^{+++} : Fe^{++}$	+0.7477
$Cu^{++} : Cu^+$	+0.455
$Cyt\ c\ Fe^{+++} : Cyt\ c\ Fe^{++}$	+0.26

Methylene blue ox. : M.b. red.   + 0.01

Old yellow enzyme ox. : O.y.e. red.   − 0.12

Acetic acid + CO_2 : pyruvic acid   − 0.70

Extensive tables of redox potentials are available
(1) and use of these values makes it possible to know in
advance the effect of one system on another and the
completeness of reaction to be expected.

Potentiometric titration (27) shows that the complete
range of a system from fully reduced to fully oxidized
or vice versa is about 0.15 volt for two electron transfer
systems and about 0.30 volt for systems where one electron
is transferred. These are very useful facts when comparing
the effect of one system on another.

In case the ratio of oxidant to reductant in a
system is not unity the resulting potential is expressed
by the following relationship:

$$E = E_o + \frac{RT}{nF} \ln \frac{oxidant}{reductant}$$

WHERE:

E_o = Normal electrode potential

R = Gas constant 8.315 joules

T = Temperature Kelvin

n = Number of electrons involved

F = 1 faraday = 96.500 coulombs.

ln = $\log_e$

The effect of hydrogen ion on a system necessitates an additional term in the above equation, as follows:

$$E = E_. + \frac{RT}{nF} \ln \frac{(oxidant)}{(reductant)} + \frac{RT}{F} \ln (H^+)$$

Tables usually contain the E_o' value which is the potential for systems with equal concentrations of oxidant and reductant, and at pH 7 and $30°C$.

The equation $\triangle F = \frac{nFE}{4.18}$ calories per mole is the link connecting thermodynamic and redox potentials.

2. Activation Energy

From purely thermodynamic considerations a reaction should proceed spontaneously if calculations indicate a release of free energy i.e., if $\triangle F$ is minus. However it may be found that the reaction does not occur spontaneously or at best the rate is infinitesimal. This condition indicates an energy barrier, that is the reactants require activation before reaction can occur. The full requirement of this energy of activation can be supplied by some means, such as heat or radiation, or the requirement can be lowered by catalysis.

A catalyst usually activates reactants by adsorbing them, this condensed state allowing close continuous contact. The activation also possibly involves a change in configuration of the reactants to make the reactive site more accessible by opening solvation shells, etc.

Enzymes are biochemical catalyst and will be discussed later.

If the reaction rate constants are determined at two different temperatures the Arrhenius equation can be used to calculate the energy of activation E*. The equation is:

$$E* = \frac{RT_1T_2}{T_2-T_1} \quad \ln \frac{k_2}{k_1}$$

3. Charge Transfer

An important type of redox mechanism, especially in the solid state is that of charge transfer (32). In the case of a pair of weakly interacting molecules which have appreciable orbital overlap an electron of one, if excited, can sometimes easily become associated with the other.

Another type of charge transfer is that in which an electron moves through an array of similar molecules to effect oxidation at a distance, e.g., in a photographic plate, a photosensitive dye on exposure to certain radiation releases electrons which migrate through the silver halide lattice to eventually be trapped by a speck of silver at which point reduction occurs, quite some distance from the electron source.

E. REDOX REACTIONS IN BIOLOGICAL SYSTEMS

1. Historical

Advances in biochemistry have been extremely rapid and most of them have occurred since 1920, (18). The field of oxidative metabolism has expanded at least as rapidly as

the others and an overall picture of that field is now available although many areas within the framework of oxidative metabolism are far from being completely investigated.

Although biochemical processes are governed by the same laws that apply to chemistry in general, special considerations are required for the investigation and interpretation of biochemical phenomna, namely the various reactions all occur in special environments, such as cells and interstital fluid; the reactions are coupled and interrelated in highly complex systems; the concentrations of reactants and intermediates can vary widely and at times become vanishingly small; the reactions are mediated by specific highly complex and quite labile enzymes and by hormones.

Lavoisier had thought of respiration as the union of carbon with oxygen in the lungs, but Voit in 1865 showed that many substances were formed from food before the final combination with oxygen occurred, (18). Following this, progress in the field of biological oxidation was slight until 1925 when Warburg (38) announced his theory of "oxygen activation". He had found that charcoal prepared from blood was a much more active catalyst for the oxidation of many organic compounds than was charcoal made from sugar, and attributed this activity to the iron contained in the

former. Warburg had also discovered an iron containing enzyme (now known as cytochrome oxidase or cyt a_3) which activated oxygen, and which he names "Atmungsferment" (respiratory enzyme). As the work progressed it became apparent that oxygen activation alone could not account for cellular oxidation and Wieland put forth his theory of "Hydrogen activation", proposing that oxidative enzymes activate certain hydrogen atoms of compounds so that they can be removed by a hydrogen acceptor. It was soon apparent however that this theory alone was not adequate as most metabolites can not transfer their activated hydrogen to molecular oxygen. A. Szent-Gyorgyi pointed out that both oxygen and hydrogen activation are required. It is now known that most oxidations of the dehydrogenase type involve intermediates between the donor and acceptor.

Michaelis (26) was insistant in maintaining that the fundamental concept of redox reactions must always be electron transfer regardless of any other accompanying events. e.g. in the reaction:

$$CH_3C{\overset{H}{\underset{OH}{-}}}H \xrightarrow{\quad -2e \quad -2H \quad} CH_3\overset{}{\underset{O}{C}}H$$

The two events of oxidation and dehydrogenation do not necessarily occur consecutively, but may be simultaneous. Removal of the two electrons by the oxidant sets the two hydrogens free as there is no longer sufficient bonding
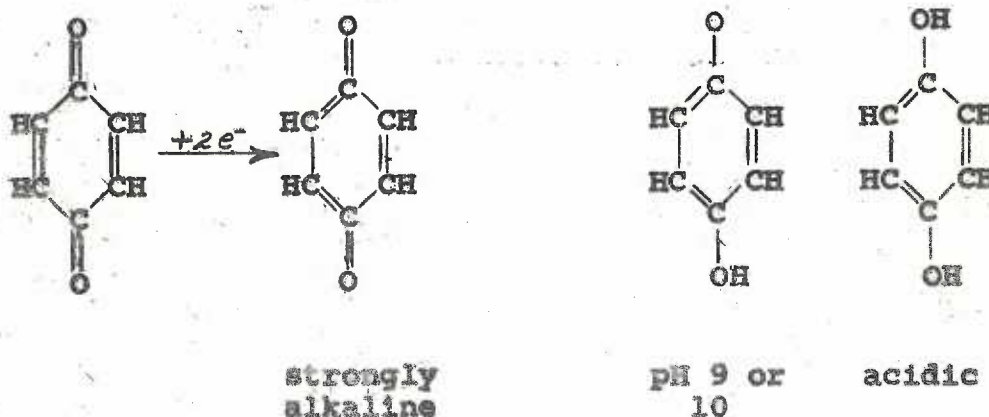
to hold them.

Also consider the oxidation of succinic acid to fumaric acid:

$$
\begin{array}{c}
\text{COOH} \\
\text{CH}_2 \\
\text{CH}_2 \\
\text{COOH}
\end{array}
\longrightarrow
\begin{array}{c}
\text{COOH} \\
\text{CH}\longrightarrow\text{H} \\
\text{CH}\longrightarrow\text{H} \\
\text{COOH}
\end{array}
$$

The CHs can be considered as infinitely strong acids.

The presence or absence of hydrogen on some molecules is not a reflection of the oxidation state but of the medium pH, e.g.

| strongly alkaline | pH 9 or 10 | acidic |

2. Enzymes

As in other biochemical fields the study of oxidative metabolism is largely a study of enzymology and elucidation of reaction pathways and mechanisms.

Most oxidation reduction enzymes contain a coenzyme prosthetic group and quite a few contain metal such as Fe, or Cu. They fall into several groups: dehydrogenases,

oxidases, peroxidases and hydrases. A large number of these enzymes have been isolated from various organisms and their functional mechanisms and places in metabolic pathways determined. Many of these enzymes function not alone, but in systems which form chains such as the terminal electron transport system or the glycolytic pathways, or in cycles such as the Krebs cycle or the β-oxidation scheme for fatty acids.

The complex oxidation-reduction enzymes function both through the apoenzyme protein and the prosthetic group.

Michaelis (27) expressing not only his own ideas but also those of Pauling (30), and LuValle and Goddard (19) proposed that the apoenzyme binds the substrate to its surface during reaction. The binding site is a template for the substrate, but some distortion of the substrate is required for fit. The energy of distortion is considered as the energy of activation present in the distorted bonds. His theory also logically attributes the locus of competitive inhibition to the binding site.

Whereas substrate specificity and activation are apoenzyme functions, the oxidative mechanism proper is probably exclusively coenzyme function.

Pyridine and flavin nucleotides are the coenzymes of dehydrogenases. In general the pyridine nucleotides are an integral part of a characterized holoenzyme; they are

quite easily removed and reattached. Often, however, they are reactants from the medium, and are bound to the protein along with the formal substrate molecule, being released and replaced at each cycle. The flavin nucleotides on the other hand are tightly bound to their protein and are usually difficult to remove.

Iron porphyrins of cytochromes are the prosthetic groups in those redox ractions which involve electron transport without hydrogen transfer, (with the possible exception of cyt b).

Oxidases effect oxidation by molecular oxygen and Mason (21) in a comprehensive review of the oxidases divided them into three major groups on the basis of function, namely, oxygen transferases, mixed function oxidases, and electron transfer oxidases.

Oxygen transferases mediate reactions in which all of the metabolized oxygen appears in the oxidized substrate. For these the known prosthetic groups are: iron and other heavy metals.

The mixed function oxidases catalyze reactions in which one-half of the utilized oxygen appears in the oxidized substrate and the other half is reduced. The prosthetic groups here are similar to those of the preceding class, i.e. heme, iron and other heavy metals.

The electron transfer oxidases produce oxidized

substrates but all of the metabolized oxygen appears either in hydrogen peroxide or water. Here the prosthetic groups are: flavin nucleotides, hemes, iron and other heavy metals and in a couple of cases pyridine nucleotides. Several of these enzymes contain more than one cofactor, e.g. xanthine oxidase contains iron, FAD * and molybdenum.

Not all mechanisms of coenzyme function are well understood. The pyridine and flavin nucleotides function through cyclic oxidation and reduction. The flavins form free radical intermediates (to be discussed later), as demonstrated by Michaelis (27) and by Beinert & Sands (4), and Bamford and Jenkins (2) discussed the theoretical and actual absence of free radicals in the pyridine nucleotide mechanisms.

The iron porphyrins evidently function in a cyclical but quite complex manner. The first assumption was of a simple valence change in the central metal atom. However, Bamford and Jenkins (2) point out that changes in the absorption spectrum during reduction of cytochrome show modification of the conjugated system. Chance and Ferguson (8) using EPR spectroscopy were able to detect free radicals in peroxidase complexes I and II, but the concentration was almost negligible, the highest being 2×10^{-7} for complex II, and they seemed to consider the results as negative. Mason and Yamazaki (23) using EPR

* Flavin adenine dinucleotide.

spectroscopy detected no free radical in the complexes I and II.

Although a great many oxidative enzymes contain metals the state of knowledge about their function is rather sketchy and far from satisfactory. Much of the evidence for their action is presumptive being based on observations, such as inactivation of the enzyme by metal binding agents such as dipyridyl in the case if iron or cyanide in the case of copper, or by dialysis. Recently, however, strong evidence has been presented by Bray et al (5) for definite valence changes in the iron and molybdenum of xanthine oxidase; and by Nakamura (29) for copper function in laccase through valence change. The function of copper in cytochrome oxidase has been a subject of rather active discussion: Wainio et al (39) and Sands and Beinert (33) present evidence of active participation by the copper, while Yonetani (42) presents evidence to show that at least 60 - 70% of the total copper is nonfunctional.

Calvin (6) described copper chelates which function like enzymes, e.g., A chelate of copper-pyridoxal-amino acid which readily carries out transamination to produce the corresponding keto acid and pyridoxamine.

Reid (32) suggested that some biological macromolecules, such as nucleic acids can provide conduction paths for reducing electrons, that is, a series of charge transfer

components to provide for oxidation at a distance. He also mentioned Calvin's hypothesis of chlorophyll acting as a charge separator battery, the stack of chlorophyll molecules, after radiant activation, providing a means of transferring the charge to one end of the stack for reducing purposes while the other end acts as an oxidant.

Integral structure is perhaps of prime importance in the function of many enzyme sequences, e.g., in the terminal electron transport chain, Green and Hatefi (15) point out that lipids (90% phospholipid) account for 30% of the dry weight of mitochondria, Co Q, vitamin E, carotenoids, and substantial quantities of cholesterol occur in the neutral lipids. Furthermore these lipids probably exist in a state of orientation which in effect makes them water soluble. The lipid composition varies but little throughout the chain, and this constant lipid protein association and the known activating effect of lipids in some catalytic functions of the protein constituents are significant indications of the essential role played by lipids in terminal electron transport. In view of the fact that fragmentation of the mitochondria produce chain segments with the same lipid structuring it is thought that the architecture is of fundamental importance both in electron transport and oxidative phospholylation.

The energy produced by respiration through the tricarboxylic-acid cycle and the terminal electron transport

chain is utilized to the extent of 40% in the production
of ATP which is used to drive most of an organisms
activities.

F. ONE AND TWO ELECTRON TRANSFER AND FREE RADICALS

Until fairly recently bivalent oxidation was a generally
accepted principle in organic chemistry. Consideration of
only the reactants and end products of organic redox
reactions produces a logical scheme of two electron transfer
for each step, e.g.

$$C_2H_5OH \xrightarrow{\frac{1}{2}O_2} CH_3CHO + H_2O \xrightarrow{\frac{1}{2}O_2} CH_3COOH$$
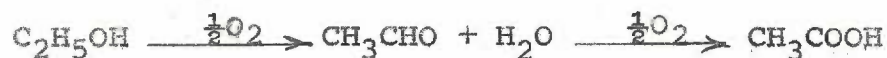
Chance mentioned that Gomberg (7), in 1900, demonstrated the
existance of the free radical triphenyl-methyl, by means of
molecular weight determinations, but the significance of such
compounds was unappreciated by most people for about thirty
years; and that Willstater, in 1904, had discovered an
intermediate in the reduction of iminoquinone but considered
it to be a molecular compound of the fully reduced and
fully oxidized states, (a meriquinone). Chance goes on to
say it was the work of Paneth and Hofeditz in 1929, on
tetraethyl-lead, and Haber and Weiss in 1934, on chain
reactions in aqueous solutions, that lead to the acceptance of
the idea of the role of free radicals as intermediates in
photochemical and pyrolytic reaction in the gas phase and
as polymerization intermediates in solution.

During the same period Michaelis built up his theory of compulsive monovalent oxidation-reduction for organic and biochemical reactions. His interest in dyes and potentiometric titration had early resulted in his observation of redox reaction intermediates as: transient colors in some dye reactions, and as inflections in some redox titration curves. Between 1930 and 1950 Michaelis wrote extensively on the subject of biological oxidation-reduction (24), (25), (26), (27), (28). Through this period he highly developed and systematized the theory and practice of potentiometric tiration. Among other things he advanced the concept of the free radical formation constant, and showed that the potentiometric method will not detect free radical when the constant of free radical formation is down to about 10^{-2}, which represents a concentration of about 0.005 of the system, i.e., 0.5%

The two monovalent step redox theory was expressed in a logical straight forward series of steps (26): The theory states that all organic oxidations, although appearing bivalent proceed in two successive steps, the intermediate being a free radical, whether it is very unstable, fairly stable or perfectly stable. He reminds us that stability is a relative matter dependent on environment, e.g., pyrogallol in alkaline solution is readily oxidized when oxygen is present, but is stable in the absence of oxygen; and formaldehyde in alkaline solution undergoes the Cannizzaro reaction, namely

$$2 \text{ HCHO} \xrightarrow{\text{NaOH}} \text{CH}_3\text{OH} + \text{HCOONa}$$

an internal redox reaction. This latter reaction is the type that accounts for the instability of free radicals and is characteristic of one step organic oxidation which produces the apparent bivalent oxidations; e.g., duroquinone if reduced in alkaline solution is converted from a faintly yellow to a colorless compound. There is, however, a fleeting brown intermediate, the free radical.
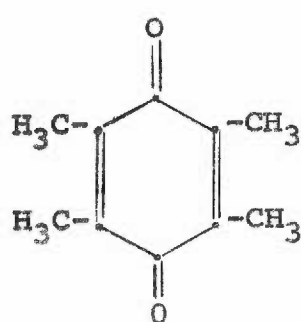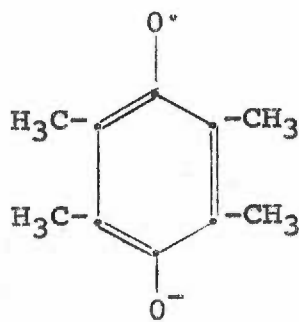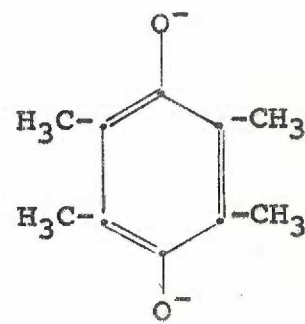
Q	SQ	QH$_2$
Faint Yellow	Brown	Colorless

In this reaction equilibrium is established instantaneously unlike the usual formation of equilibria in organic reactions, there is no sluggishness, the activation energy being extremely small.

When duroquinone is reduced in alkaline solution there is at all times, except at the beginning and end, a mixture of
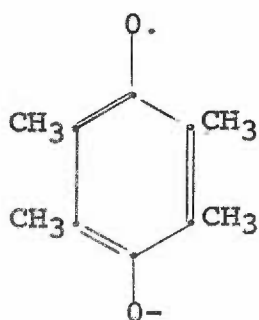
Q, SQ and QH$_2$

in reversible equilibrium and the equation

$$2SQ \rightleftharpoons Q + QH_2$$

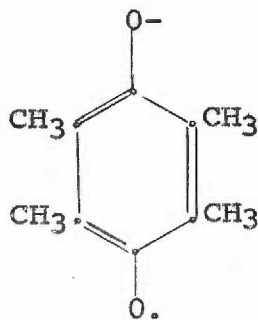is implicit. The semiquinone formation constant can be expressed

$$K = \frac{[SQ]^2}{[Q][QH_2]},$$

and the reciprocal is the disproportionation constant. K is large in alkaline solution and small in acid solution.

In alkaline solution   In acid solution

I   II

Form I is stabilized by resonance while form II is nonresonant on the oxygen and so less stable than II.

The redox problem is diphasic; the speed is a kinetic consideration while the completeness of reaction is thermo-dynamic dependent.

The thermodynamic aspect is expressed in the redox potential tabulations in which the members reduce other members above them and oxidize those below them.

The kinetic aspect can be viewed as thermodynamic as follows: with reversible redox reactions equilibrium is established almost instantaneously. But with irreversible reactions this is in general not the case, and a relatively large quantity of activation energy is required - the condition which prevents the random haphazard occurrence of so many thermodynamically possible reactions, in fact the condition which makes possible the existance of organic chemistry.
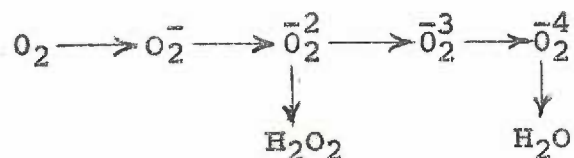
In the irreversible type reaction with its high requirement of activation energy, the concentration of free radicals is small and there is a continuous dismutation. Thus the velocity of reaction is dependent on the square of the free radical concentration and a large activation energy is required for formation of the free radical, whereas with reversible redox reactions the free radical is far more stable and the activation energy for its formation is low. Furthermore free radicals can dimerize,

$$2FR \longrightarrow D$$

resulting in a lower active concentration. In other words activation energy is mainly required for free radical formation in the first step of the two step oxidation process, and is

low for reversible and high for irreversible reactions. Inhibition due to high activation energy requirement occurs only when the attainable concentration of free radical is the limiting factor for the overall reaction rate.

The foregoing explains the sluggishness of oxygen as an oxidizing agent despite its high oxidizing potential. In univalent steps:

$$O_2 \longrightarrow O_2^- \longrightarrow \overset{-2}{O}_2 \longrightarrow \overset{-3}{O}_2 \longrightarrow \overset{-4}{O}_2$$
$$ \downarrow \phantom{\longrightarrow \overset{-3}{O}_2 \longrightarrow} \downarrow$$
$$ H_2O_2 \phantom{\longrightarrow \overset{-3}{O}_2 \longrightarrow} H_2O$$
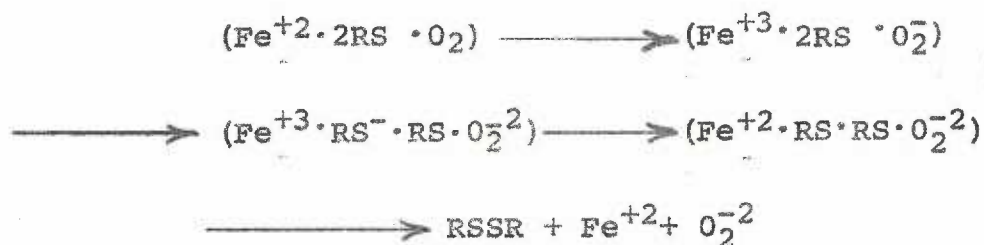
Steps 2 and 4 are unstable and require high activation energies, which in nonphysiological conditions, such as combustion, can be furnished by direct heat to produce free radicals, but in reactions such as the oxidation of cysteine catalysis is require, e.g., by a transition metal such as iron or copper. If a solution of cysteine (RSH) containing some Fe^{++} is exposed to the oxygen of air a purple color first forms, then fades out, and subsequent analysis shows that the cysteine has been oxidized to cystine (27). The iron has two properties which make possible this catalysis: first, it readily changes valence between two and three, i.e., Fe^{++}, and Fe^{+++}, and second, it forms Werner type complexses.

These reasons, of course, also apply to the other transition metals acting as catalysts. Returning again to the catalyzed oxidation of cysteine: the iron has six coordination sites, but will combine with only two cysteine molecules, due perhaps to the large size of the cysteine molecule and steric hindrance, each cysteine is attached to the ferrous ion at two points, that is between the cysteine carboxyl and one of the ferrous valence bonds, and between the cysteine amino nitrogen and one of the nonionic coordination points of the iron by using the unshared election pair of the amino nitrogen.

$$Fe^{+2} + 2RSH \longrightarrow Fe(RS)_2 + 2H^+$$

The resultant complex $Fe(RS)_2$ still has two available coordination sites and can form stable compound, such as $Fe(RS)_2(CO)_2$. However, attempt to form a similar compound with oxygen in place of carbon monoxide fails to produce a stable complex and instead oxidation of the cysteine occurs, the reaction is presumed to proceed as follows:

$$(Fe^{+2} \cdot 2RS \cdot O_2) \longrightarrow (Fe^{+3} \cdot 2RS \cdot O_2^-)$$

$$\longrightarrow (Fe^{+3} \cdot RS^- \cdot RS \cdot O_2^{-2}) \longrightarrow (Fe^{+2} \cdot RS \cdot RS \cdot O_2^{-2})$$

$$\longrightarrow RSSR + Fe^{+2} + O_2^{-2}$$

The Fe^{+2} can now go through the cycle again and the O_2^{-2} perhaps forms hydrogen peroxide possibly by the reaction

$$2RSH + O_2^{-2} \longrightarrow RSSR + H_2O_2$$

The H_2O_2 can then oxidize an additional two molecules of cysteine.

The high activation energy requirement of oxygen protects oxyhemoglobin from oxidation.

Oxidation of cysteine by iron in the above example is an intramolecular redox reaction, which type is possibly characteristic of many redox reactions.

1. Properties Of Free Radicals

Free radicals have two distinguishing charateristics: paramagnetism, and in general extremely high chemical reactivity; both resulting from the possession of an unpaired electron. Ingram (17) defines a free radical as "a molecule, or part of a molecule, in which the normal chemical binding has been modified so that an unpaired electron is left associated with the system". This definition excludes certain occurrences of unpaired electrons; namely, the transition metal ions and molecules such as O_2. Both characteristics of unpaired electrons, extreme chemical reactivity and paramagnetism, have been used to detect and identify them. As mentioned earlier, Gomberg was the first to demonstrate the existence of free radicals by isolating triphenylmethyl;

the most convincing proof of its free radical nature being

its high chemical reactivity (17). A number of other

workers have used chemical techniques to identify free

radicals, e.g., the mirror technique demonstrated by Paneth

and Hofeditz in 1929 (17).

2. Detection Of Free Radicals

Physical methods of detecting unpaired electrons include

the Gouy balance (17), which measures paramagnetic

susceptibility in a very direct manner, and electron

paramagnetic resonance spectroscopy (EPR spectroscopy),

first developed by E. Zavoisky (44). The principles of the latter

are herewith presented in a nonrigorous manner.

An electron has, in addition to its orbital quantum

values, an intrinsic energy of spin on its axis, which spin

has the quantum value $S = \pm 1/2$. When associated with another

electron, as in a normal chemical bond, the electrons are

oriented so that their spins cancel, thus reaching a lower

energy level and greater stability. In the case of a free

radical species the unpaired electrons have their magnetic

moments in randon order, i.e., pointing in all directions, and

all of equal value. If a D.C. magnetic field is applied to

such a random assortment, the unpaired electrons will

aggregate into two groups, one with spins and magnetic moments

parallel to the magnetic field and the other with spins

and magnetic moments antiparallel to the magnetic field.

The two groups are at different energy levels; those with

parallel alignment having a value of 1/2 gβH less than they had at zero field value while those with antiparallel alignment have a value of 1/2 gβH greater than they had at zero field value. These conditions can be diagrammed as follows:
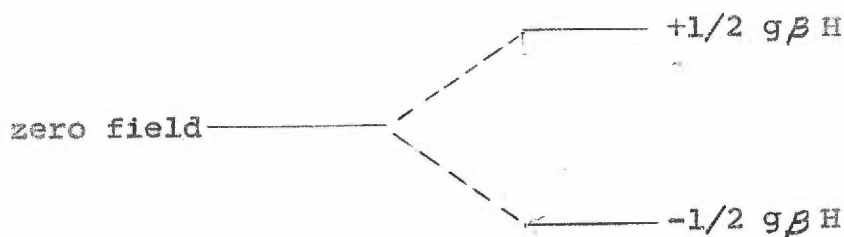
g, called the g-value or spectroscopic splitting factor, is the contribution of the electrons spin and orbital motion to its total angular momentum, and is equal to 2.0023 for completely free spin.

β is the Bohr magneton $= \frac{eh}{4\pi mc} = 5585$ ergs gauss^{-1}mole^{-1} and converts angular momentum to magnetic moment.

H is the field strength in gauss.

The total splitting is thus gβH and is directly proportional to the value of the applied magnetic field.

Distribution between the two energy levels is not quite equal and is given by the Maxwell-Boltzmann equation:

$$\frac{n_1}{n_2} = e^{-\frac{\Delta E}{KT}}$$

n_1 = number of unpaired electrons in upper state

n_2 = number of unpaired electrons in lower state

ΔE = separation between levels

K = Boltzmann constant

T = absolute temperature

At room temperature the ratio, $n_1 : n_2$ = 0.9984

As can be seen from the equation, lowering the temperature results in greater splitting.

If a split system of unpaired electrons is irradiated at a frequency ν such that $h\nu = g\beta H$, some electrons in the lower state will absorb energy and jump to the upper level, and at the same time some of those in the upper state will be stimulated to emit radiation and fall to the lower level. It is absorption of energy at the lower level which is measured by the EPR spectrometer. The process of absorption occurs more readily than that of emission, and if emission was the only mechanism for loss of energy by electrons in the upper level, saturation would soon occur, absorption would fall and an approximately static condition would soon be established. The upper level electrons can however lose energy through so-called relaxation processes. When spin energy is shared with the thermal vibration of the body as a whole, the relaxation process is called spin-lattice interaction. The spin-lattice relaxation time is

the time in which an initial excess of energy will drop to
one-half its value, and strong spin-lattice interaction
results in short relaxation time and vice versa.
Saturation can occur with relatively low microwave power if
relaxation time is quite long, i.e. in the order of seconds.

The relation $h\nu = g\beta H$ must be established if a
spectrum is to be observed, and theoretically   and H
could be taken at any set of values which satisfy the
equation. However, in microwave work, it is necessary to
operate at a fixed frequency because of the narrow frequency
range of kylstron tubes which are used to generate the
microwave beam, and the rigid mechanical components, namely,
the wave guide and resonance cavity. Therefore, it is the
field strength which is varied to fulfill the equation
requirements with different g-values.

As shown by the equations presented above, the
sensitivity of the instrument is increased by lowering the
temperature, and by increasing the magnetic field strength
and microwave frequency. The intensity of absorption
varies as ν^2. However, practical considerations limit the
field strength, as it is necessary to apply a very uniform
field to an approximately 1 cc space. The upper limit
attainable by the best magnet design is about 10,000 gauss.
The microwave frequency used is, of course, fixed by the
field strength in order to satisfy the fundamental equation.

Most of the current EPR spectrometers use a mean field of
about 3 K gauss and a klystron generated microwave of about
3 cm (about 9000Mc/sec.)

It is perhaps advisable at this point to give an
elementary description of the actual EPR instrument
and its functioning.
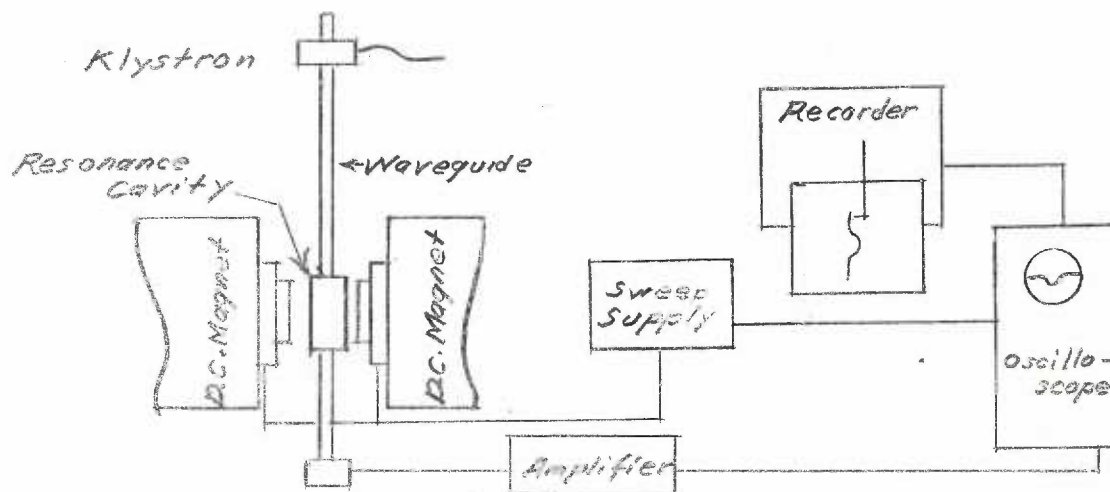
The klystron generates a fixed microwave frequency
which is transmitted along the waveguide to the resonance
cavity in which the sample is located. The resonance cavity
is so constructed that a high level of microwave power is
concentrated at its center which is also the sample's
position. The two large D.C. electromagnets generate the
magnetic field which passes through the cavity and sample.

The D.C. power supply to the electromagnet is varied through a regular cycle to produce a cyclic variation of the D.C. magnetic field. This is the so-called magnetic sweep of the field and provides the continuous variable H to satisfy the fundamental equation $h\nu = g\beta H$ for all values of g. After passing through the sample the klystron beam is picked up by a crystal detector, the output of which is amplified and fed to an oscilloscope and recorder.

The instrument is operated so that the sweep current is in phase with the x-plates of the oscilloscope while the detector output is imposed on the y-plates. The recorder tape is driven at a constant speed and the pen simply reflects variations in the detector output.

If the sample in the resonance cavity contains unpaired electrons (such as those of a free radical or transition metal) they will be segregated into two energy states separated by the value $g\beta H$ when irradiated by the energy input.

The field is continually varying and as it passes through the value which satisfies the fundamental equation energy absorption occurs, less energy reaches the detector, the detector output drops, and the falling current from the detector is reflected as an excursion of the oscilloscope and recorder traces. As H passes the critical value absorption falls off and ceases, the detector power rises, and the traces return to their neutral positions thus completing the signal.

In actual practice indirect methods of detection are employed to increase sensitivity and reduce the noise to signal ratio. One method is superheterodyne detection in which a secondary frequency is imposed on the regular microwave beam and the resultant frequency is detected and amplified. Another method is the balanced hybrid tee in which the crystal detector is in one branch of the wave guide to the resonance cavity and the klystron is at the end of another branch, the whole system being in electrical balance. Absorption of energy in the resonance cavity leads to imbalance which is reflected in the crystal detector.

Also in actual practice the magnetic field is continually modulated so that it samples only a small part of the free radical distribution curve and is in fact continually measuring the slope of the curve. The recorded signal is therefore the first derivative of the distribution curve.

When the orbit of an unpaired electron is within effective proximity of an atom possessed of a nuclear spin and magnetic moment, there is interaction between the electron and nucleus. The nucleus has a quantum spin value of $I = \pm 1/2$ and a resulting magnetic moment of $M_I = \pm 1/2$, and the electron also has a quantum spin value of $S = \pm 1/2$ and a resulting magnetic moment of $M_S = \pm 1/2$. If the two moments have the same sign the electron's energy level will rise while if the signs are different, the electrons energy level will fall. The unpaired electron population is thus split into two energy levels. This effect

is called hyperfine interaction, and the resultant change
in EPR signal is referred to as hyperfine splitting.

This situation in an applied magnetic field can be
diagrammed thus:
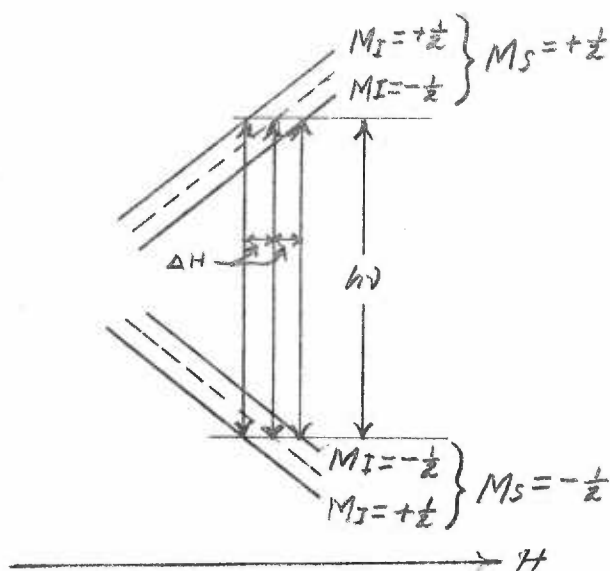
There thus results two secondary energy distributions
in both the upper and lower main levels. When irradiated with
resonant microwave power only the electron spins will change
orientation, the nuclear spins will be unaffected, and so
the only transitions will be between levels which have the
same quantized component of nuclear spin, namely:

$$(M_I=+1/2)(M_S=+1/2) \text{ and } (M_I=+1/2)(M_S=-1/2), \text{ or}$$
$$(M_I=-1/2)(M_S=+1/2) \text{ and } (M_I=-1/2)(M_S=-1/2)$$

It is thus apparent two transitions will occur at magnetic
fields given by

$$H_1 = \frac{h\nu}{g\beta} - \Delta H \qquad\qquad H_2 = \frac{h\nu}{g\beta} + \Delta H$$

The number of lines caused by hyperfine splitting
depends on the number of nuclei with which the electron
interacts and the relative strength of interaction. For
equal interaction with n nuclei the number of lines is
given by $(2nI+1)$, but since $I=1/2$ the number of lines under
this condition is $(n+1)$. As shown above interaction with
one nucleus produces a two line spectrum, interaction
with two nuclei produces three lines, etc.

Equal interaction with nuclei is also characterized
by a binomial distribution of line intensity, e.g., when
electrons in the upper energy level with magnetic moment
$M_S=+1/2$ interact with two nuclei they are split into two
levels by the first nuclear magnetic moment $M_{I_1} = \pm 1/2$,
and these groups are again split by the second nuclear
magnet moment $M_{I_2} = \pm 1/2$ into two levels, thus
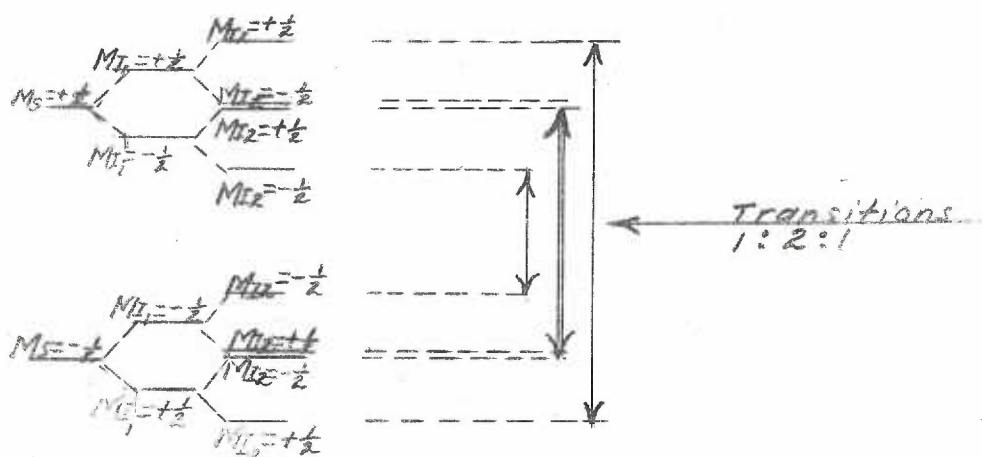
Since the $M_{I_2}=-1/2$ subcomponent of the $M_{I_1}=+1/2$
component is coincident with the $M_{I_2}=+1/2$ subcomponent
of the $M_{I_1}=-1/2$ component there can be but three energy
transitions and hence three lines in the spectrum.
The center line represents on the average, twice as
many transitions as either of the other two and is therefore
twice as intense so that the distribution and intensity
formula is 1:2:1, showing three lines with the center of
twice the intensity of the other two. The levels of the
central transition are double degenerate. For interaction

of an unpaired electron with the four equivalent nuclei
of a p-benzosemiquinone molecule the resulting spectrum will
be 1:4:6:4:1.

The foregoing referred to conditions of equal interaction
with nuclei, but if the electron interacts more effectively
with one nucleus than with another the spectrum is complicated
by two unequal splittings, the stronger interaction causes a
splitting that produces $(2I_1+1)$ well separated lines, each of
which is further split into $(2I_2+1)$ subcomponents by the
weaker interaction.

Hyperfine splitting provides much information about
the molecule with which an unpaired electron is associated.

a. Quantitative Aspects

EPR spectra in addition to being qualitative expressions
of free radical species are very dependable quantitative
expressions. To determine a free radical concentration, it is
only necessary to perform a double integration of its EPR
spectrum to obtain the area under the true distribution curve.
This area can then be compared with that of a known
concentration of free radicals whose spectrum has been obtained
under the same conditions as the spectrum of the unknown.
Several stable free radical species are available as standards,
1,1- diphenyl -2- picryl-hydrazyl being much used at present,
has the following structural formula:
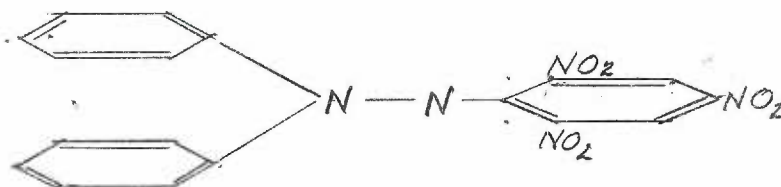
It is possible to calculate free radical concentration
from the change in power occuring at the detecting crystal,
provided certain other characteristics of the spectrometer are
accurately known.   This is, however, a difficult procedure and
is rarely used.

The present lower limit of sensitivity for EPR equipment
is about 10" unpaired electrons per gram at millisecond
intervals.

EPR spectroscopy is now being employed in various
types of chemical investigation, e.g., irradiation induced
changes in material, polymerization, pyrolysis, photochemical
reactions, and the detection of free radical intermediates
in biochemical redox reactions.   Such studies include not
only demonstration of free radical mechanisms, but also
identification of the free radical species, elucidation of the
reaction mechanisms and solutions of kinetic problems.

3. Applications

Use of EPR spectroscopy dates from the work of Commoner,
Townsend and Pake in 1954 (12) in which they found free
radicals present in a variety of biological materials.

In 1954, Commoner et al (9) observed a large increase in the free radical content of tobacco chloroplasts when illuminated, and a subsequent decay in the dark, and surmised that the signals were due to either or both an excited state of chlorophyll and redox reaction intermediates.

Around 1958 Commoner and Lippincott (11) showed that the free radical produced by illumination of FMN is the same as that obtained on its reduction, either with inorganic reducing agents or DPNH. They then extended the study to flavoprotein enzymes. Commoner et al (10) observed typical flavin free radical formation when the enzyme lactic oxidative decarboxylase acted on its substrate.

At about the same time Ehrenberg and Ludwig (14) by means of EPR spectroscopy confirmed the occurrence of a free radical when Warburg's old yellow is reduced with TPNH.

Vanngard, Bray, Malmstrom and Pettersson (37) using anaerobic titration and EPR spectroscopy, and Bray, Pettersson and Ehrenberg (5) using anaerobic titration and both EPR spectroscopy and spectrophotometry observed signals for FMN, iron and manganese when xanthine oxidase acted on its substrate.

Beinert and Sands (3) in 1959, followed the anaerobic titration of cyt c reductase with DPNH by means of EPR spectroscopy and observed that about 28% of the total iron was reduced and then was reoxidized on exposure to air; also that subsequent to the iron reduction a flavin-free radical

signal appeared. The flavin but not the iron could be reoxidized by DPN, and they concluded that the electron flow sequence probably was : substrate $\longrightarrow$ flavin $\longrightarrow$ iron.

In 1959, Yamazaki, Mason, and Piette (41) (42) applied EPR spectroscopy and a flow system in a study of peroxidatic oxidation of ascorbic acid, dihydroxy-fumaric acid and hydroquinones and observed, for the first time, free radicals in enzyme catalyzed oxidation of substrate and in addition studied the reaction kinetics.

Mason, Ingram, and Allen (22) found free radicals to be intrinsic components of the various melanins.

Nakamura (29) by using an EPR spectrometer with the field strength set at the peak of the semiquinone spectrum, and employing a flow system was able to study the kinetics of laccase oxidation of hydroquinone and prove that the semiquinone is the first product formed.

The foregoing are but a few of the more important research projects in biochemistry which have been made possible by the concept of EPR spectroscopy.

G. THE ENZYME LACTIC OXIDATIVE DECARBOXYLASE (LOD)

This enzyme from Mycobacterium phlei was reported by Edson (13), and from M avium by Yamamura at al (40).

It was shown to catalyze the oxidation of lactate to acetate and CO_2, and FAD was suggested as the prosthetic group.

Sutton (34) using M phlei isolated and partially purified the enzyme but could find no evidence of riboflavin. He found the reaction to be an oxidation and decarboxylation:

$$CH_3CHOHCOOH + O_2 \longrightarrow CH_3COOH + CO_2 + H_2O$$

Later, Sutton (35) with further purification of the enzyme, found that a brown hemin pigment absorbing at 405 to 415 mu had obscured the riboflavin prosthetic group, and he could, after its removal, follow the oxidation process spectrophotometrically. By means of reactivation, experiments and paper chromatography, he found that the prosthetic group is similar to, if not identical, with riboflavin-5'-phosphate, (F M N).

Using O_2^{18} atmosphere, Hayaishi and Sutton (16) showed that one atom of atmospheric oxygen is incorporated into the new carboxyl group and one into water:

$$CH_3CHOHCOOH + O_2^{18} \longrightarrow CH_3COO^{18}H + CO_2 + H_2O^{18}$$

Sutton (36) crystallized the enzyme and determined the molecular weight as about 260,000, and the equivalent weight as 125,700 showing that there are two F M N prosthetic groups.

Catalase caused no decrease in the quantity of oxygen used indicating that no H_2O_2 is released in the reaction and carbonyl fixatives, e.g., hydroxylamine, detected no pyruvate, so if H_2O_2 and pyruvate are intermediates, they are held at

the protein surface for the final reaction to form acetate,
CO_2 and H_2O. As previously mentioned, Commoner et al,
using E P R spectroscopy followed the reaction of L O D on its
substrate. He concluded that the steady state concentration
of free radical varies with the rate of electron transport.
This was on the basis of finding that the free radical
concentration increased with increasing lactate up to a plateau,
then leveled off.

Beinert and Sands (4) working with L O D spectro-
photometrically observed formation of the intermediate when
the enzyme acted on lactate.

1. Thesis Problems

Determination of a free radical formation constant for
L O D was the object of the major research project described
in this thesis.

Also described herein are the results of a survey of a
limited number of foodstuffs for the purpose of detecting free
radicals and where present making an estimate of concentration.

II METHODS AND MATERIALS

A. Determination Of A Free Radical Formation Constant

1. Technique Theory

The free radical concentration of the riboflavin -5'-phosphate groups in L O D should be at a maximum when they are 50% reduced. Commoner and Lippencott (11) found that FMN free radical is readily formed when the flavin is reduced with a suitable reducing agent. They also demonstrated that oxygen rapidly reoxidizes the flavin prosthetic groups of L O D with consequent loss of the free radical.

Consideration of these facts indicated that it would be necessary to titrate the enzyme anaerobically with a reducing agent while following the changing concentration of free radical by means of E P R spectroscopy, to the 50% reduced condition.

$$Q \longrightarrow SQ \longrightarrow QH_2$$

but;

$$SQ + SQ \longrightarrow Q + QH$$

2. Instruments

E P R spectra were obtained with a Varian V - 4500 x - band spectrometer Fig. 1. Included in the auxiliary apparatus is a liquid nitrogen cooled circulating system for the resonance cavity, and a thermocouple for accurate temperature determinations.

The anaerobic spectrometer titration cell, Fig.2, has an overall height of about 27 cm and consists essentially of three parts: The lower narrow portion of the main body is a quartz tube 10 cm long with an inside diameter of 3 mm. It is sealed at the bottom and is joined to the upper portion of the main body by a graduated joint consisting of several grades of glass to prevent breakage on contraction and expansion. The upper part of the main body is pyrex and has a diameter of about 10 mm and contains a stopcock with an 8 mm opening. The side arm, also of pyrex, is about 5 mm in diameter and contains a stopcock. The side arm permits evacuation of the apparatus followed by nitrogen flushing, and the large opening in the main body stopcock permits easy insertion of the titration pipette and stirring rod during nitrogen flushing. It is thus possible to open the apparatus for titration and still maintain an anaerobic condition. This instrument was patterned after one used by Beinert and Sands (4).

A special assembly, here called "the nitrogen chamber" was convenient both for standardizing the reducing agent against FMN under anaerobic conditions, and as a pressure buffer vessel when admitting nitrogen to the evacuated spectrometer cell. It consists of a large test tube, approximately 4 cm in diameter and 15 cm long fitted with a two-hole rubber stopper. Within the large test tube is a smaller one approximately 1.5 cm in diameter and 10 cm long; setting on a cotton pad. A 1 cm

diameter glass tube extends through one hole in the rubber stopper and for several centimeters into the smaller test tube. This tube has a large-opening stopcock incorporated into its upper end. A 4 mm. diameter glass tube extends through the other opening of the rubber stopper and down into the large test tube, but outside of the smaller test tube, and also has a stopcock incorporated into its upper end. This nitrogen chamber was evacuated and flushed with nitrogen through the smaller glass tube while the larger glass tube was used for insertion of pipettes into the smaller test tube which served as a titration vessel. The nitrogen chamber is shown in Figs. 3 and 7.

The micropipette used for measuring exact volumes of enzyme suspension and FMN solution is a capillary glass tube about 25 cm. in length, and was graduated by weighing the volume of water contained between two marks. The intended volume was 0.2 ml. but actual calibration showed it to be 0.2088 ml. Fluid was drawn into and expelled from the pipette by means of a syringe attached to one end by Tygon tubing. This pipette can be seen in Figs. 4 and 7.

The titration pipette, syringe and micrometer drive can be seen in Fig. 4. The pipette is a piece of capillary glass tubing drawn to a fine tip at the lower end while the upper end is fastened with Tygon tubing to a Hamilton #1002, 2.5 ml. gas tight syringe. The syringe barrel is held by

the clamp of a Radiometer micrometer, the plunger being advanced by the micrometer. The micrometer drive was calibrated from the weight of water expelled from the syringe by various movements of the drive. The value of one vernier division is 0.00042 ml.

A Carey recording spectrophotometer was used to determine the concentrations of enzyme, FMN and 1,1-diphenyl-2-picryl-hydrzyl.

A Beckman Zeromatic pH meter was used for buffer adjustment.

3. Reagents

The lactic oxidative decarboxylase was generously furnished by Dr. W. B. Sutton. The silver oxide was made by Dr. H. S. Mason.

Item	Manu-facturer	Lot No.
FMN	Mann	10881
Sodium dithionite	Baker	90835
8 hydroxy-quinoline	Matheson, Coleman and Bell	2759
Sodium pyrophosphate	Baker	3850
Benzene	Baker	M 100J
1,1 diphenyl-2-picryl hydrazine	Eastman	7365

All water used was distilled and deionized.

All nitrogen used was prepurified and hydrocarbon-free.

All buffer used was 0.1 M sodium pyrophosphate, pH 5.9 prepared as follows: 0.1 mole of $Na_4P_2O_7 \cdot 10 \ H_2O$ was dissolved in 950 ml H_2O, the pH was lowered to 5.9 with concentrated HCl, the volume was increased to 1 liter and the pH was verified.

An EPR spectrum of the buffer prepared from regular analytical grade pyrophosphate showed the presence of iron, and it was therefore considered necessary to prepare iron free sodium pyrophosphate, as follows:

Sixty g of $Na_4P_2O_7 10H_2O$ crystals were ground to an impalpable powder with a mortar and pestle. The powder was placed in an Erlenmeyer flask, and 50 ml 8-OH-quinoline 2% in $CHCl_3$ was added; the flask, was shaken vigorously for several minutes and within ten minutes of mixing the pyrophosphate was recovered by centri-fuging in an International centrifuge. This procedure was performed twice, followed by placing the salt in a vacuum dessicator overnight. The treated $Na_4P_2O_7 \cdot 10H_2O$ was washed with several volumes of $CHCl_3$ in a Buechner filter and again placed in the vacuum dessicator. The treated pyrophosphate was tested for Fe by means of thioglycolic acid ($HSCH_2COOH$) in dilute ammonia, which detects 1 part in 10 million, and found to be Fe-free. Thereafter all buffer used was made up as described above using the iron-free sodium pyrophosphate.

One, 1-diphenyl-2-picrylhydrazyl was used as a primary standard of free radical concentration and was prepared from the corresponding hydrazine as follows:

One hundred mg. 1-1 diphenyl-2-picrylhydrazine were dissolved in 50 ml C_6H_6, 5g Ag_2O were added to the solution (in a 150 ml Erlenmeyer flask), a magnetic stirring bar was introduced, the flask was sealed, placed on a stirring base, and allowed to stand for 48 hours with moderate stirring. The mixture was filtered through Whatman #42 filter paper to remove Ag, and Ag_2O.

The DPPH as shown above was synthesized in benzene and the EPR spectra on this standard were run with benzene as a solvent. However, the only recorded spectra found (35) were for DPPH in chloroform. Some DPPH made by Dr. G. Narni and recrystalized from chloroform was used to determine the molecular extinction coefficient in the following manner:

1. $\log \in$ shown in literature= 4.27 at 330 mu.

2. $\in$ shown in literature= 18,621.

3. $\in$ of Narni's DPPH in $CHCl_3$ = 14,434.7

4. Purity of Narni's DPPH = $\dfrac{14,434.7}{18,621}$ = 0.77522

5.   of Narni's 77.52% DPPH in C_6H_6 13,243.7

6.   of Narni's DPPH @ 100% conc. $\dfrac{13243.7}{0.77522}$ = 17084

o.d. of the DPPH used as a standard in this project = 0.78 and the concentration is therefor:

7. $\dfrac{0.78}{17084}$ = 4.5655×10^{-5} M. = 0.0457 mM.

The concentration of FMN solutions were determined by measuring the optical density at 450 mu.

4. Procedures

Three complete anaerobic titrations of the LOD enzyme were run, and were designated Experiments I, II and III. Experiment I which was an orientation procedure employed Sutton's preparation 273-4-254A (9-20-60) which contained 46.95 mg of enzyme ml^{-1} The volume used, 0.2088 ml contained 9.8032 mg of protein.

Sutton's preparation No. 273-4-255 was used in both Experiments II and III. The preparation contained 71.55 mg of enzyme ml^{-1}, and 0.2088 ml or 14.9396 mg of protein were used in each experiment.

Description of a complete anaerobic titration.

Experiment II is herewith described in detail to illustrate the procedure and techniques involved.

a. Preparation of the dithionite solution. 50 ml of iron free sodium pyrophosphate buffer o.1 M., pH 6.9 in a 150 ml erlenmeyer flask was vigorously infused with nitrogen through a gas scrubber tube for about five minutes before, during and subsequent to the solution of sufficient sodium dithionite to produce an approximately 5mM solution (an excess of dithionite was always added). Two gas tight syringes were immediately drawn full of the dithionite solution while it was still being infused with nitrogen.

b. Standardization of the sodium dithionite solution.
1 ml of 306uM FMN solution (made up in iron-free sodium
pyrophophate buffer 0.1M, pH 5.9 and standarized
spectrophotometrically from   o.d. at 450 mu) was placed
in the inner test tube of the nitrogen chamber. The chamber
was progressively evacuated with a vacuum pump and then
connected to a tank of nitrogen gas for continuous flushing.

A glass capillary titration pipette was attached to one
of the sodium dithionite filled syringes and about 1 ml of the
dithionite was expelled through the tip to flush out all air.
The syringe barrel was then clamped in the micrometer drive
frame and the titrating pipette was lowered into the FMN
solution. A polyethylene capillary tube with its opening at
the bottom of the FMN solution furnished an auxiliary stream
of nitrogen gas to stir the solution during titration and to
further insure an anaerobic condition.

During titration the FMN solution was irradiated with
ultraviolet light and fluoresced with a brilliant yellowish
green color until the end point was reached at reduction of
the FMN, when fluoresence ceased. Fig. 3 shows the
arrangement for standardization of the dithionite. Repetition
of the titration showed no significant difference in the
dithionite titer, 0.0396 ml dithionite was required for
reduction of the 1ml of 306uM FMN solution. The dithionite
is therefor $\frac{304 \times 10^{-6} M}{396 \times 10^{-2}}$ or 7.676 mM.

The concentration of the dithionite was not used in calculating the free radical formation constant, but instead served as a guide to the approximate amount of dithionite which would be required for 50% reduction of the enzyme. It was in effect a guard against passing the end point, as any variation of requirement would always be on the side of a larger dithionite requirement because of its instability.

c. Anaerobic titration of the enzyme with EPR monitoring of free radical level.

The empty anaerobic spectrometer titration cell was placed in the resonance cavity of the EPR spectrometer, Fig.5, and the spectrum was run to check for contamination.

The cell was then removed from the resonance cavity, and using the measuring micropipette, 0.2088 ml of Sutton's LOD preparation No. 273-4-255, containing 14.9396 mg of the enzyme, was placed in the bottom of the quartz tube part of the cell. The cell was evacuated, immersed in liquid nitrogen, placed in the resonance cavity of the spectrometer and scanned. The spectrometer settings for the entire titration were: amplitude modulation 32 gauss, gain 200 and scan rate 264 gauss/min., and the temperature of the resonance cavity was -138 C.

The cell was removed from the cavity, thawed, connected to the nitrogen tank and continuously flushed with nitrogen; during which the titration pipette was lowered into the

enzyme and 0.00084 ml of the standardized sodium dithionite was injected, the titration pipette was removed and the enzyme solution stirred by the vertical motion of a 1 mm diameter glass stirring rod. The stopcocks of the tube were closed, the nitrogen was disconnected and the tube was immersed in liquid nitrogen, placed in the resonance cavity and scanned.

This procedure was repeated for each addition of dithionite and the titration was carried up to the maximum free radical concentration and then along the ensuing curve until it had practically leveled off. All increments of dithionite were 0.00084 ml until the titration was well past the critical point.

The three experimental curves are shown in Fig. 6.

The arrangement of apparatus employed to flush the spectrometer cell with nitrogen is shown in Fig. 7. By using a wye connection in the nitrogen line it was fairly easy to release some of the pressure through the nitrogen chamber while opening and closing the stopcocks on the spectrometer cell. Without some such arrangement there is danger of a pressure build-up blowing off the tube connection to the spectrometer cell and allowing air contamination.

As mentioned in the general discussion of EPR spectra, free radical concentration is measured by comparing areas under distribution curves; the EPR signals being first derivatives of the corresponding free radical distribution curves.

Also as mentioned in the "reagents" section the
primary standard chosen for these experiments was
1, 1-diphenyl-2- picrylhydrazyl, hereafter referred to
as DPPH.

EPR spectra of DPPH at various temperatures from
25 C to -138 C, showed that the free radical spectra
vary in an abnormal manner at temperatures below -120 C,
Fig. 8a. This behavior dictated the necessity of employing
a secondary standard that would show a regular progressive
increase in signal size with decreasing temperatures,
throughout the required range. The so-called carbon
standard, a "pitch" material was chosen and its spectrum
was obtained at 25 C and -138 C to provide a connecting
link between the DPPH standard at 25 C and the reduced
LOD at -138 C, Fig. 8b.

B. Free Radical Survey of Some Foodstuffs

1. Instruments

EPR spectra were obtained with the Varian V-4500
x-band spectrometer listed under Instruments in Part I.
The spectrometer cells used were long (30 cm.) quartz tubes
3 mm i.d. closed on one end.

2. Reagents

The foodstuffs surveyed fall into several categories,
namely, beverages, cereals, condiments, fish and meats,
fruits and vegetables. A complete list of the items is
included in the "Results" section.

3. Procedures

Enough of each item to cover the "swept space" was placed in the cell, and the cell was then placed in the spectrometer resonance cavity and scanned. If the material was moist or if it was liquid, the cell was immerced in liquid nitrogen prior to placing in the resonance cavity, which in this case was also at liquid nitrogen temperature.

Figure 1. The Varian V-4500 X-band
electron paramagnetic
resonance spectrometer.
The cabinet on the far
left is the D.C. magnets'
power supply; in the
center the control console
with: detecting, sweep and
recording units; and on the
right the D.C. magnets.
Immediately above the magnets
is the cabinet which houses
the klystron tube and its tuning
equipment. The wave guide is
seen extending from this
cabinet to the resonance cavity,
located between the magnet
pole faces.

Fig. 1   58.
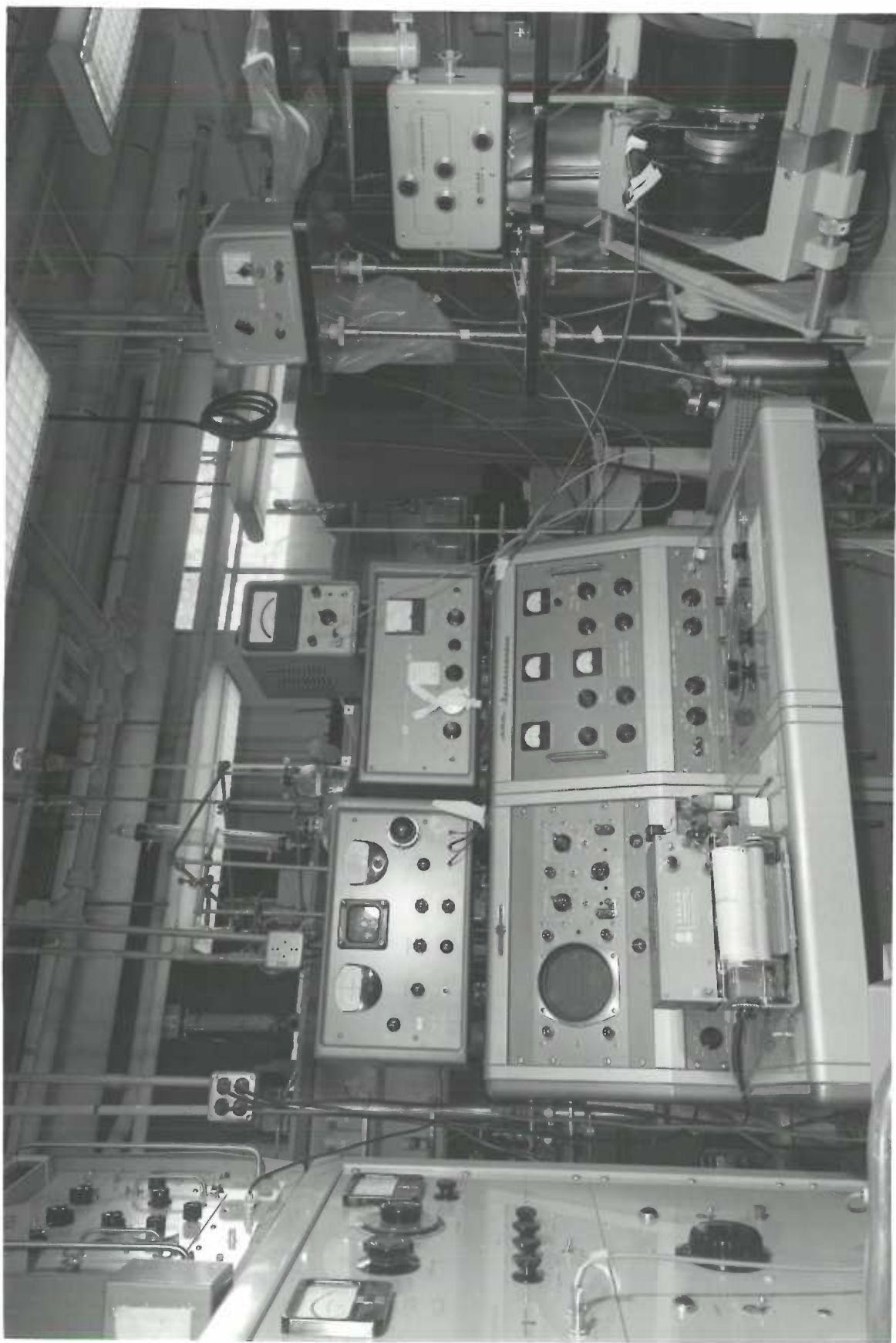
Figure 2. The anaerobic spectrometer
titration cell. The
overall height is about 27 cm.
The lower, narrow part is
the spectrometer cell
proper, and is a quartz
tube 10 cm. long with an
inside diameter of
approximately 3 mm. The
part of the apparatus above
the quartz tube is pyrex.

Fig. 2.

60.
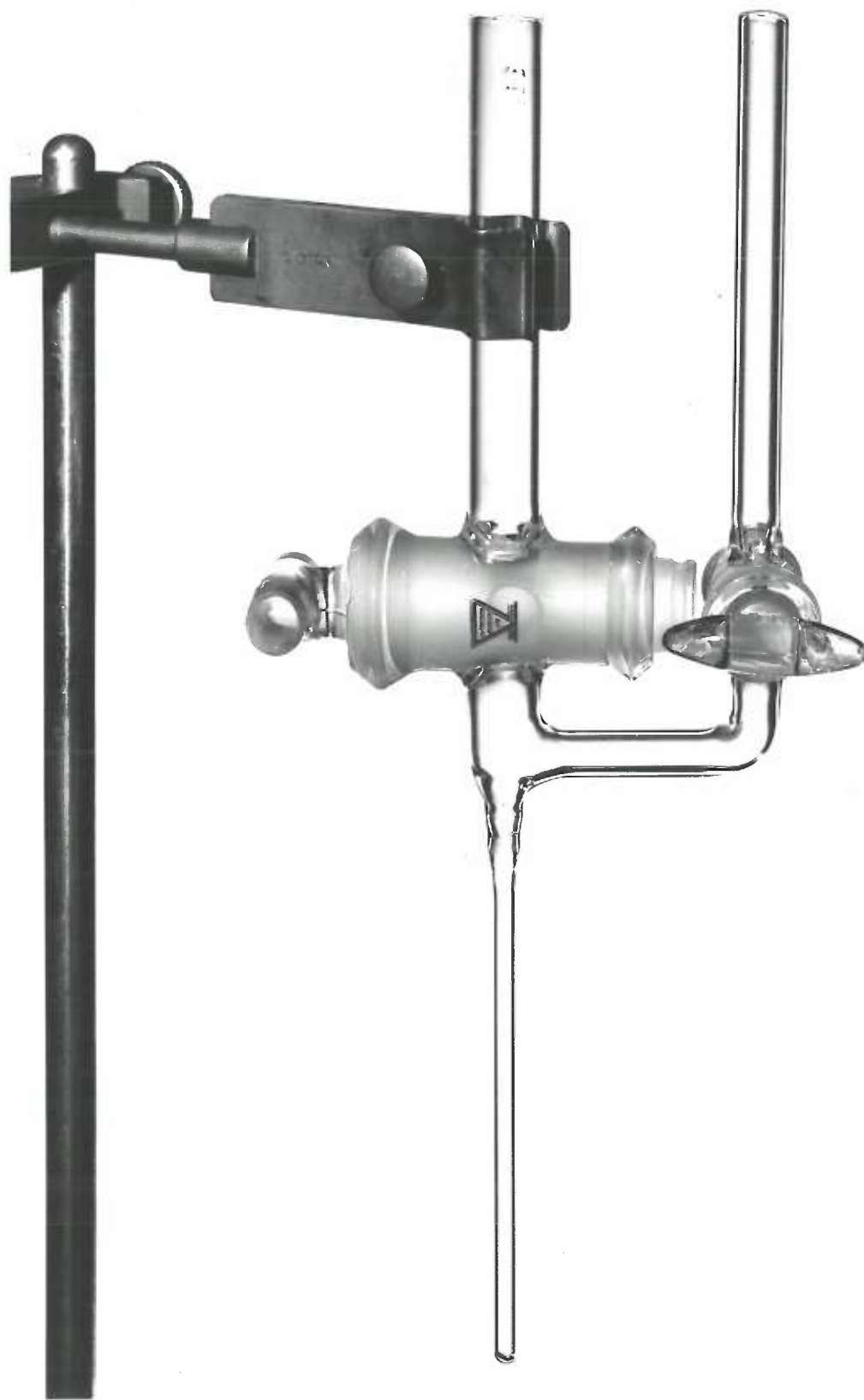
Figure 3.   The nitrogen chamber.

It is the large test tube with
the rubber stopper and stopcocks.
The inner chamber (the smaller
test tube) holds the FMN accessible
from the exterior; but an anaerobic
condition prevails because of
rapid nitrogen flow out through
the opening.   The nitrogen inlet
is located back of the larger
stopcock.

The polyethylene capillary
provides an auxiliary stream
of nitrogen to stir the FMN and
to further insure an anaerobic
condition.

It is shown here in the arrange-
ment used for standardization
of sodium dithionite against FMN.

Fig. 3
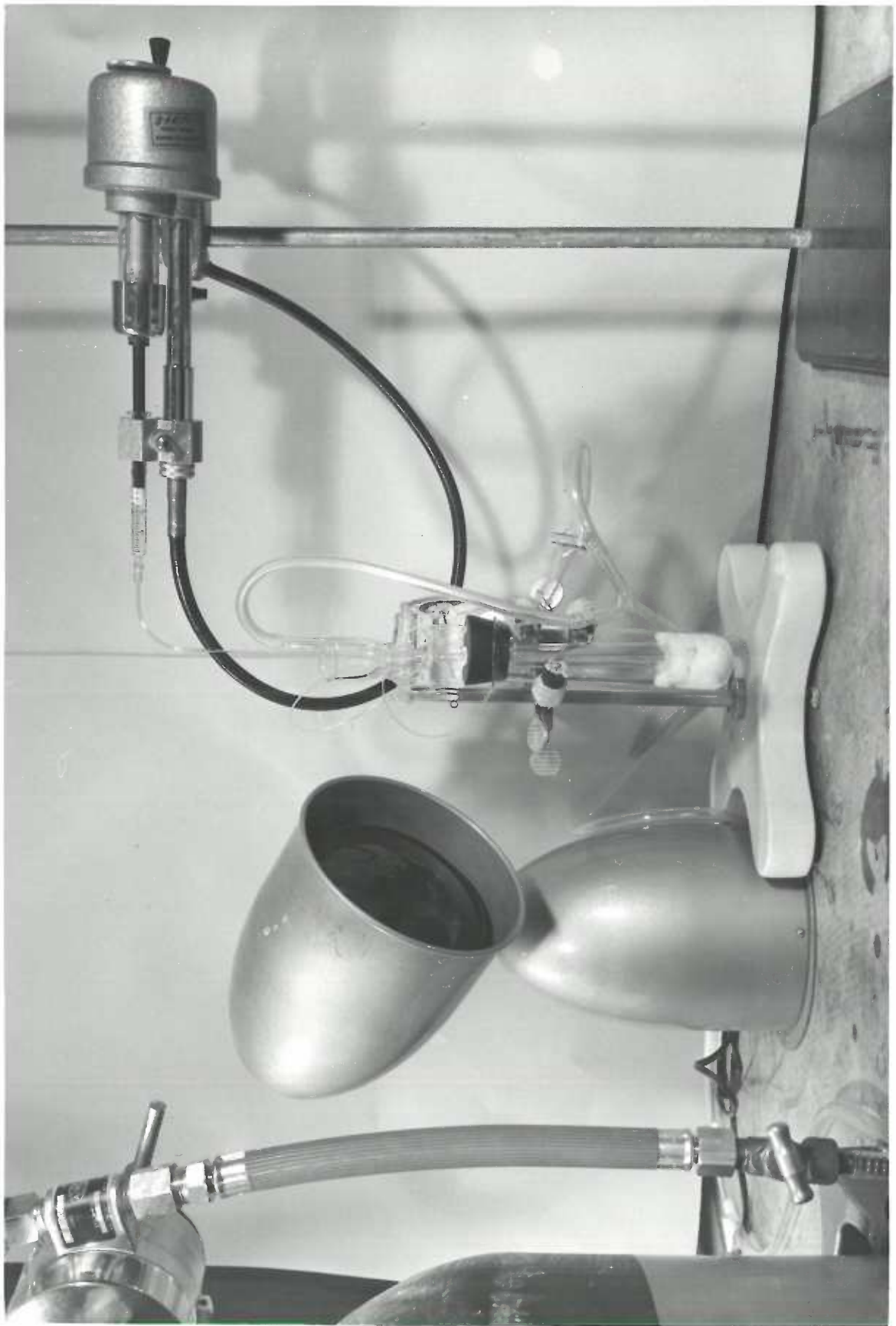
Figure 4.   The arrangement of apparatus

for anaerobic titration of

the enzyme.

The micropipette can be seen

in the lower foreground.

Fig. 4   64.
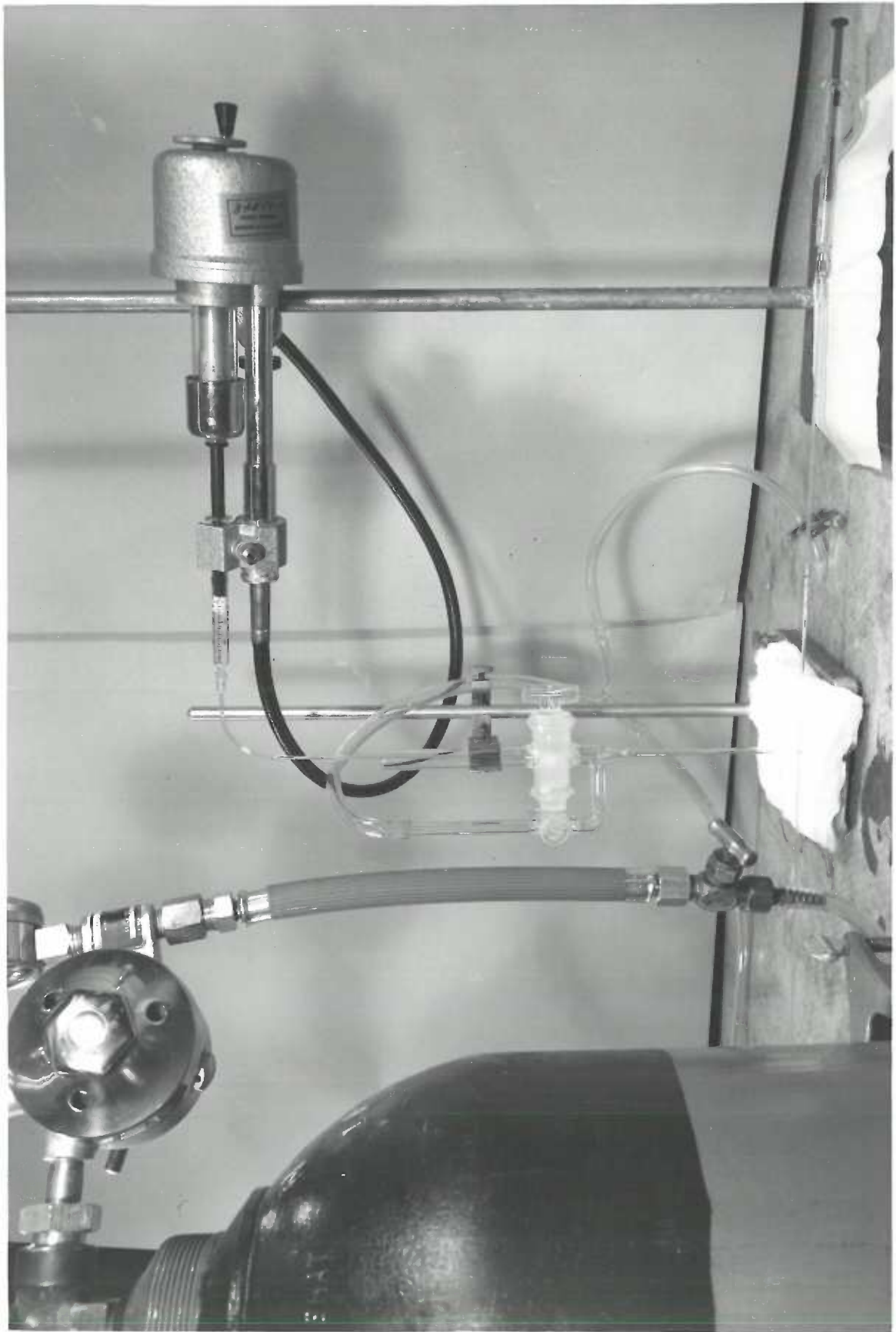
Figure 5. The anaerobic titration spectrometer
cell in the EPR spectrometer
resonance cavity.

Fig. 5
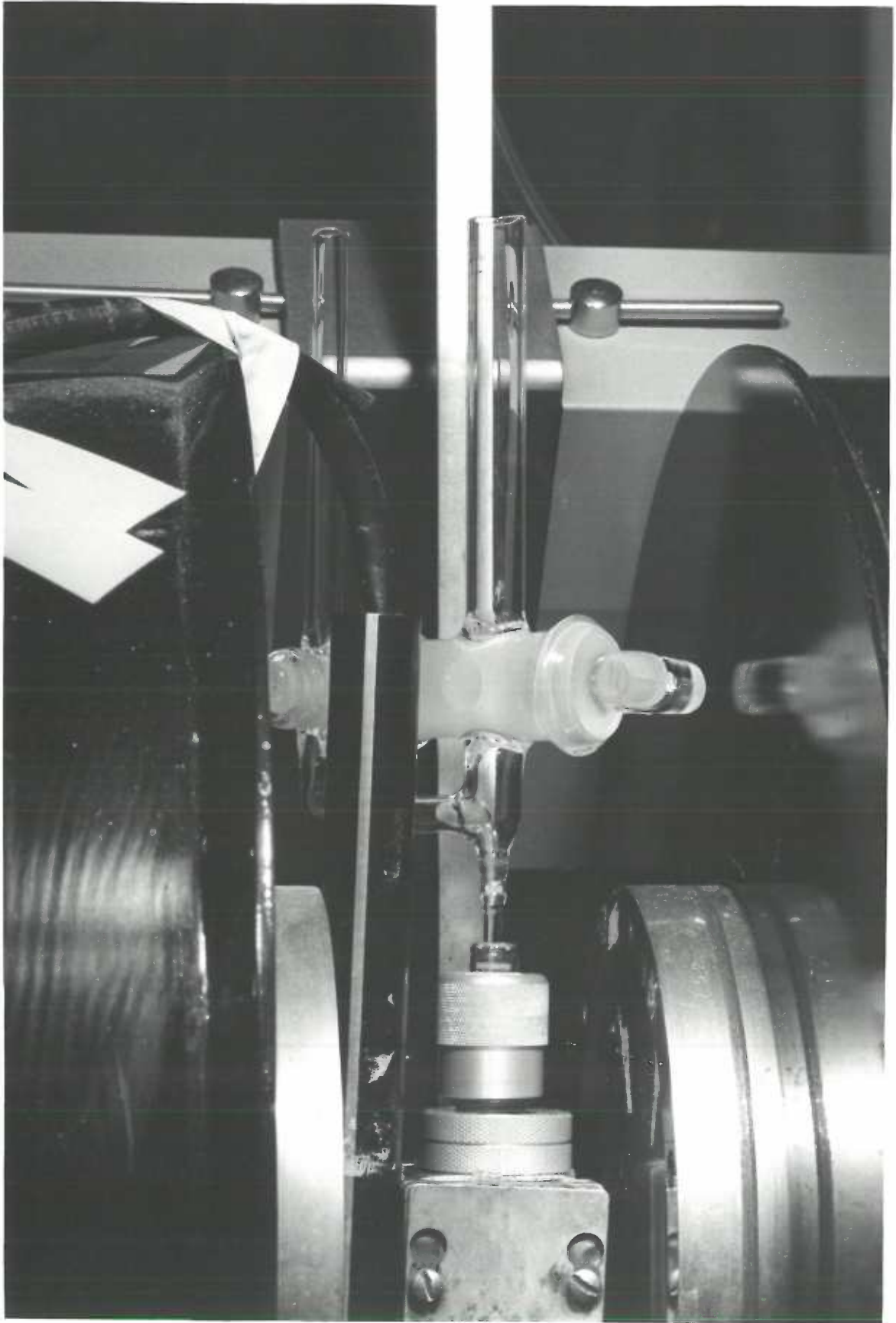
Figure 6. The anaerobic titration curves,
showing the EPR signal height
versus mu moles of sodium
dithionite; 9.8032 mg of the
enzyme were titrated in
Experiment I, and 14.9396 mg
in both Experiments II and III.

Fig. 6   68.

Fig. 6
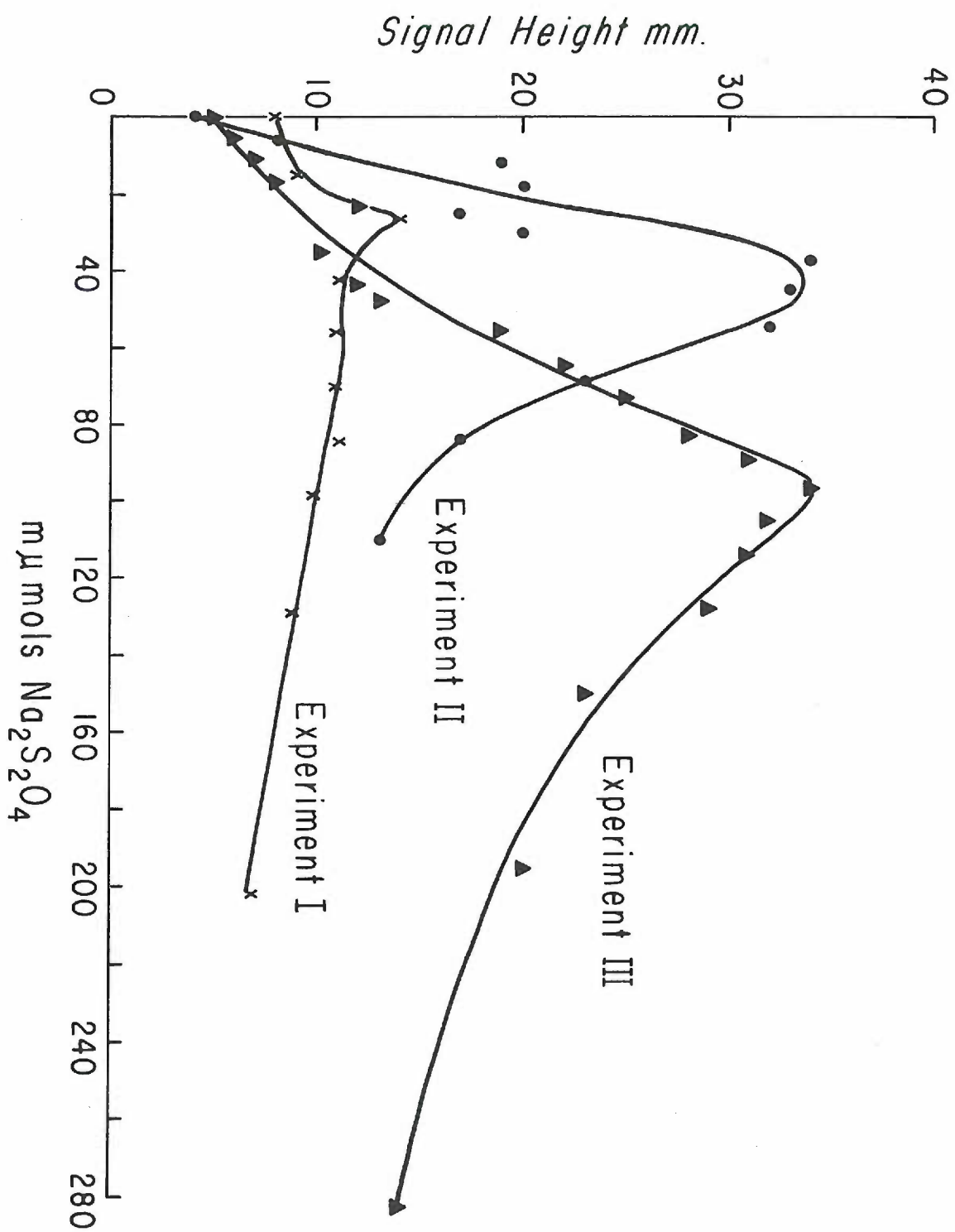
Figure 7.   Use of the nitrogen chamber

as a pressure buffer when

flushing the anaerobic titration

spectrometer cell with

nitrogen.

Fig. 7
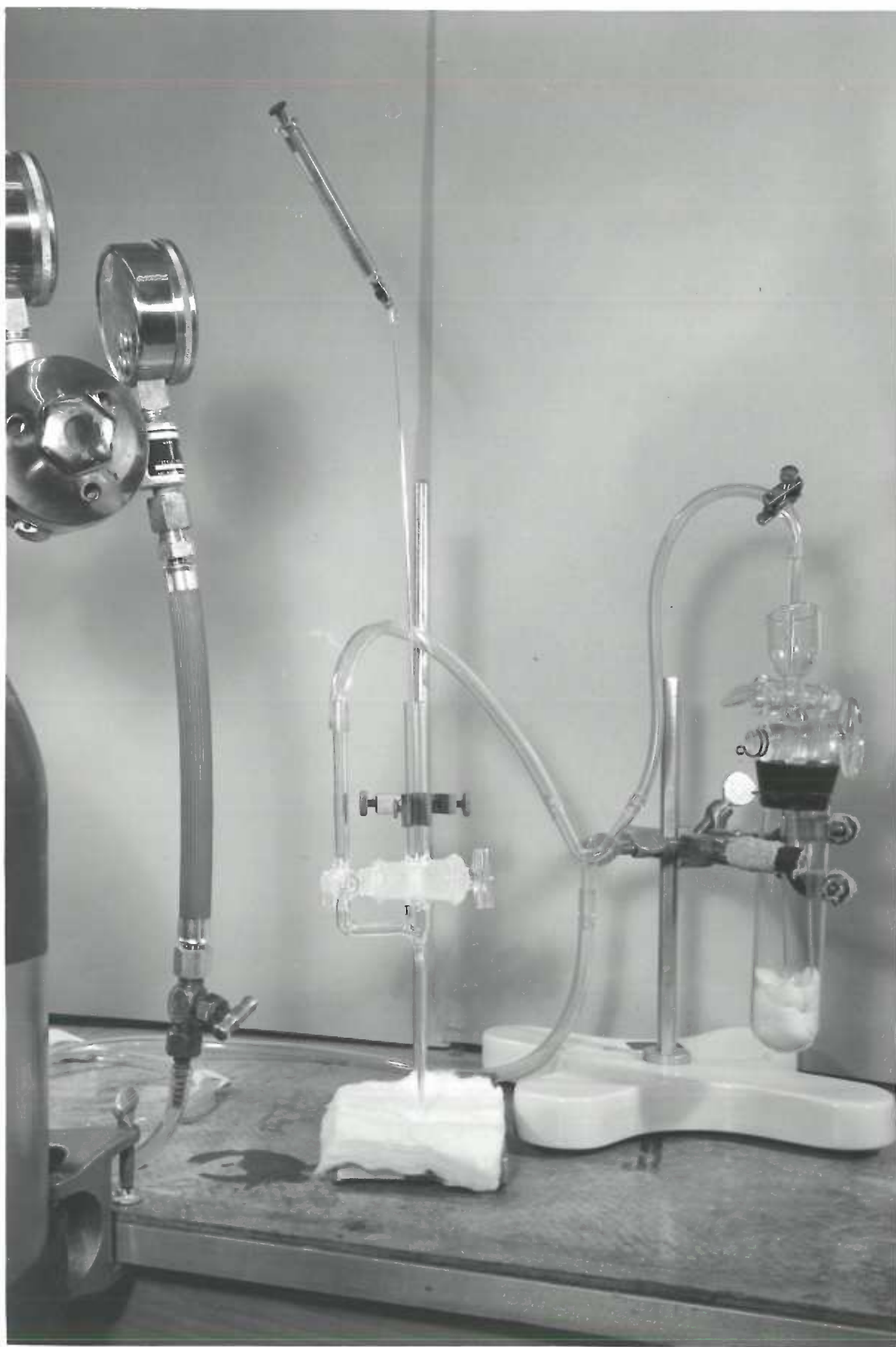
Figure 8.   a. The EPR signal heights of the 1,

1-diphenyl-2-picrylhydrazyl

standard (0.0457mM.), at

various temperatures, showing

abnormal behavior at temperatures

below -120C. Spectrometer settings:

gain = 20

scan rate = 264 gauss/min.

b. The EPR signal heights of the

carbon standard (0.0162 mM), at

various temperatures, showing smooth

change over the temperature range

covered. Spectrometer settings:

gain = 1

scan rate = 58 gauss/min.

Fig. 8.   72.

Signal Height mm.

Signal Height mm.

Temperature °Kelvin

DPPH Standard 0.0457 mM.   Gain = 20

Carbon Standard 0.0162 mM.   Gain = 1

a.

b.
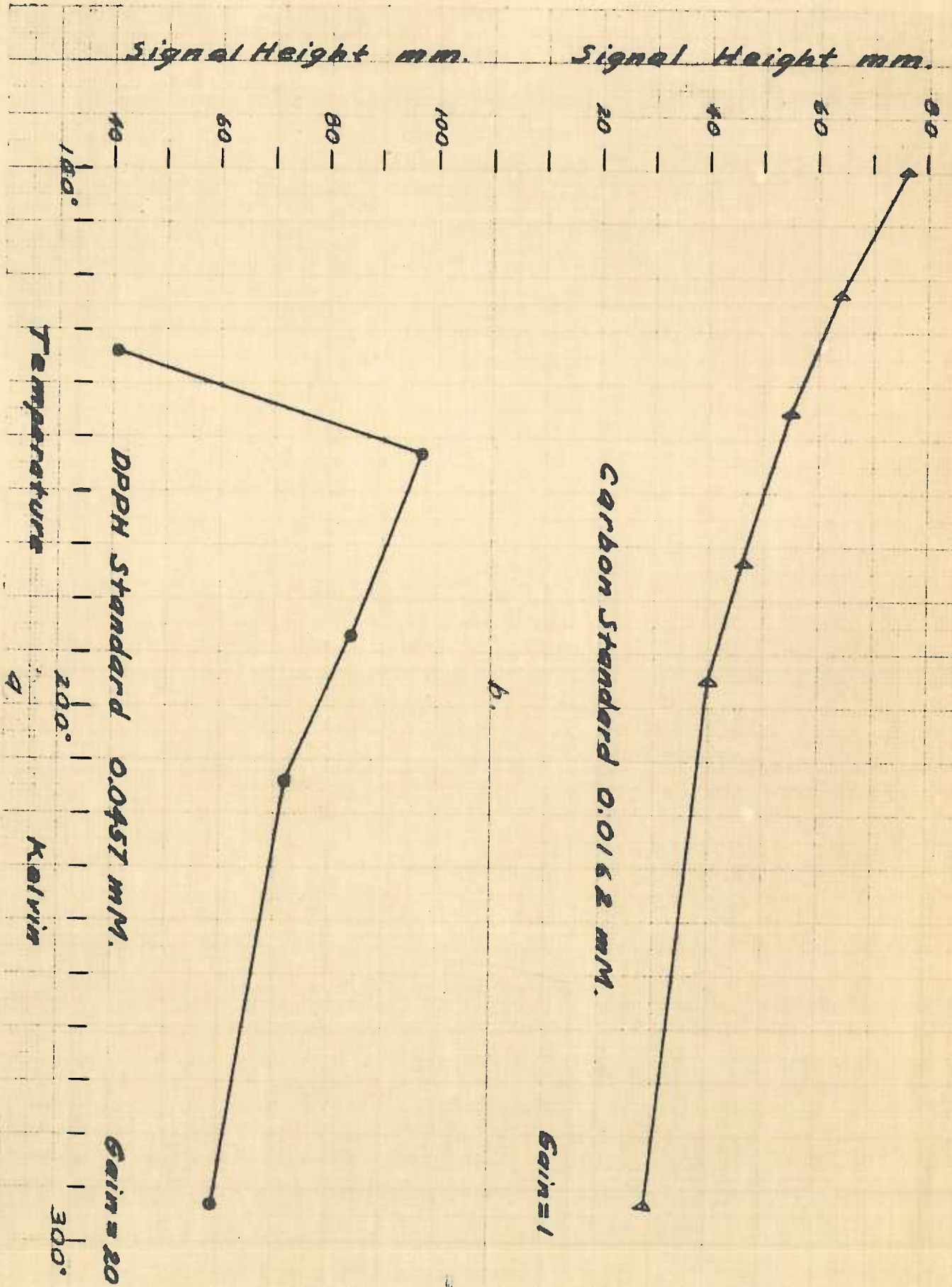
III RESULTS AND CALCULATIONS

A. Determination Of A Free Radical Formation Constant

Fig. 6, a graphical representation of Experiments I, II and III, shows the variation of EPR signal height with the quantity of reducing agent present. As previously mentioned 9.8032 mg. of the LOD enzyme were used in Experiment I while 14.93996 mg. of enzyme were used in each of Experiments II and III.

The critical value is the point at which the flavin groups of the enzyme are 50% reduced, and is the point of maximum free radical concentration, reflected in the spectrum as maximum signal height.

Experiment I is not considered in the calculations of the constant. As stated earlier it was an orientation experiment to determine the feasibility of the procedure.

The critical values obtained from Experiments II and III are identical, and were used to compute the constant.

The difference in width of the two titration curves, Fig. 6, is of no significance for the purpose of this research and is merely a reflection of the comparative anaerobic conditions in the two titrations. The steeper slopes and narrower width of the titration curve of Experiment II indicates that a more anaerobic condition existed than that of Experiment III. This resulted in a sharper response to the reducing agent in Experiment II, but could not affect the total free radical

concentration at the point of 50% reduction of the flavin groups, which point is simply arrived at sooner in Experiment II than in Experiment III.

Fig. 9 shows the series of EPR spectra for Experiment II.

The free radical formation constant is derived from the relationships:

$$(1) \qquad 2Q \underset{-2e^-}{\overset{+2e^-}{\rightleftharpoons}} 2sQ$$
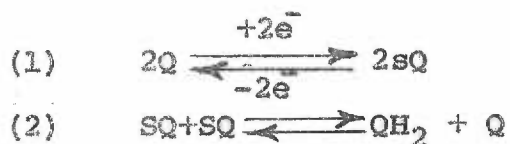
$$(2) \qquad SQ+SQ \rightleftharpoons QH_2 + Q$$

but, 2SQ is the free radical concentration, and if it is subtracted from the original quinone concentration the remainder is the sum of QH_2 and Q at equilibrium. The free radical formation constant is then expressed as:

$$K = \frac{[SQ][SQ]}{[QH_2][Q]} = \frac{[SQ]^2}{[QH_2][Q]}$$

The reciprocal of K is the dismutation constant.

Calculations from Experiment II are herewith presented. The quantity of LOD used in Experiment II was 14.93 mg, but as there was some loss of volume each time the enzyme was stirred it was necessary to correct for the loss of LOD up to and including the critical point.

Measurement of the volume remaining at the end of titration, and including in the calculations consideration of the dithionite added indicated a loss of 1.514% each time the

cell was opened and stirred. There were up to and including
the critical point 6 titration points, which left 0.1905 ml of
LOD, whereas 0.2088 ml had been present at the start.

The molecular weight of the enzyme is 260,000 and the
sample used contained 71.55 mg per ml or 71,550 mg per liter.
The sample was therefore, $\frac{71,550}{260,000} = 0.27519$ mM, or 275.19 uM, and
contained 275.19 mu moles of enzyme per ml.

The volume of enzyme at the critical point, 0.1905 ml
contained $(0.1905)(275.2) = 52.43$ mu moles of enzyme.

The primary DPPH standard was 0.0457 mM. (all free radical).
The EPR spectrum of the standard at a gain of 2.5 and a scan
rate of 110 gauss/min and at 25°C was found upon double integration
to have an area of 1223 mm^2, Fig. 10 a and b.

In like manner the carbon standard under the same conditions
was found to have an area of 433 mm^2. Therefore, the free
radical concentration in the carbon standard equals $\frac{(433)(0.0457)}{1223}$
= 0.0162 mM, Fig. 10 c and d.

The area of the carbon standard curve at a gain of 2.5,
but at a scan rate of 264 gauss/min and at -138°C. was found
to be 381 mm^2, Fig. 11 a and b.

The enzyme EPR spectrum was obtained at a gain of 200, a
scan rate of 264 and at -138°C. and double integration of the
spectrum at the critical point gave an area of 206 mm^2,
Fig. 11 c and d.

To compare the enzyme free radical concentration with

that of the carbon standard requires changing the area of
the carbon standard EPR curve to what it would be at the gain
used for the EPR curve of the enzyme and this involves a
factor of 80 i.e. $\frac{200}{2.5}$. This factor can be applied directly to
the area of the carbon curve. The more elaborate method of
correcting at each interval throughout the first integration
was tested, and in this case at least, produced substantially
the same result as that produced by application of the above
factor to the second integral of the carbon standard EPR
spectrum.

The LOD free radical concentration at the critical point
is therefor:

$$FR_{LOD} = \frac{(Area_{LOD})(FR_{Carbon})}{(Area_{Carbon})(80)} = \frac{(206)(0.0162)}{(381)(80)} = 1.09488 \times 10^{-4} \text{ mM}$$

Values at the critical point can be summarized as:

Adjusted volume of enzyme = 0.1905 ml.

Adjusted volume of dithionite = 0.0048 ml.

Total volume = 0.1953 ml.

$0.1953 \; x = (0.1905)(0.27519 \text{ mM})$

$x = 0.26842 \text{ mM}$ = final concentration of LOD

$[Q] + [QH_2] + [SQ] + [SQ]$ total LOD concentration = 268.42 uM.

$[SQ] + [SQ]$ = free radical concentration = 0.11 uM

$[Q] + [QH_2]$ = 268.31 uM

$[Q] = [QH_2]$ = 134.155 uM

From the equation:

$$2SQ \rightleftharpoons Q + QH_2$$

the free radical formation constant is:

$$K = \frac{[SQ]^2}{[Q][QH_2]}$$

and substituting the above summarized values:

$$K = \frac{[0.11]^2}{[134.155]^2} = \frac{0.0121}{17,997.5640} = 6.72 \times 10^{-7}$$

Figure 9. The complete series of the
electron paramagnetic
resonance spectra for Experiment
II, obtained during the
anaerobic titration of the
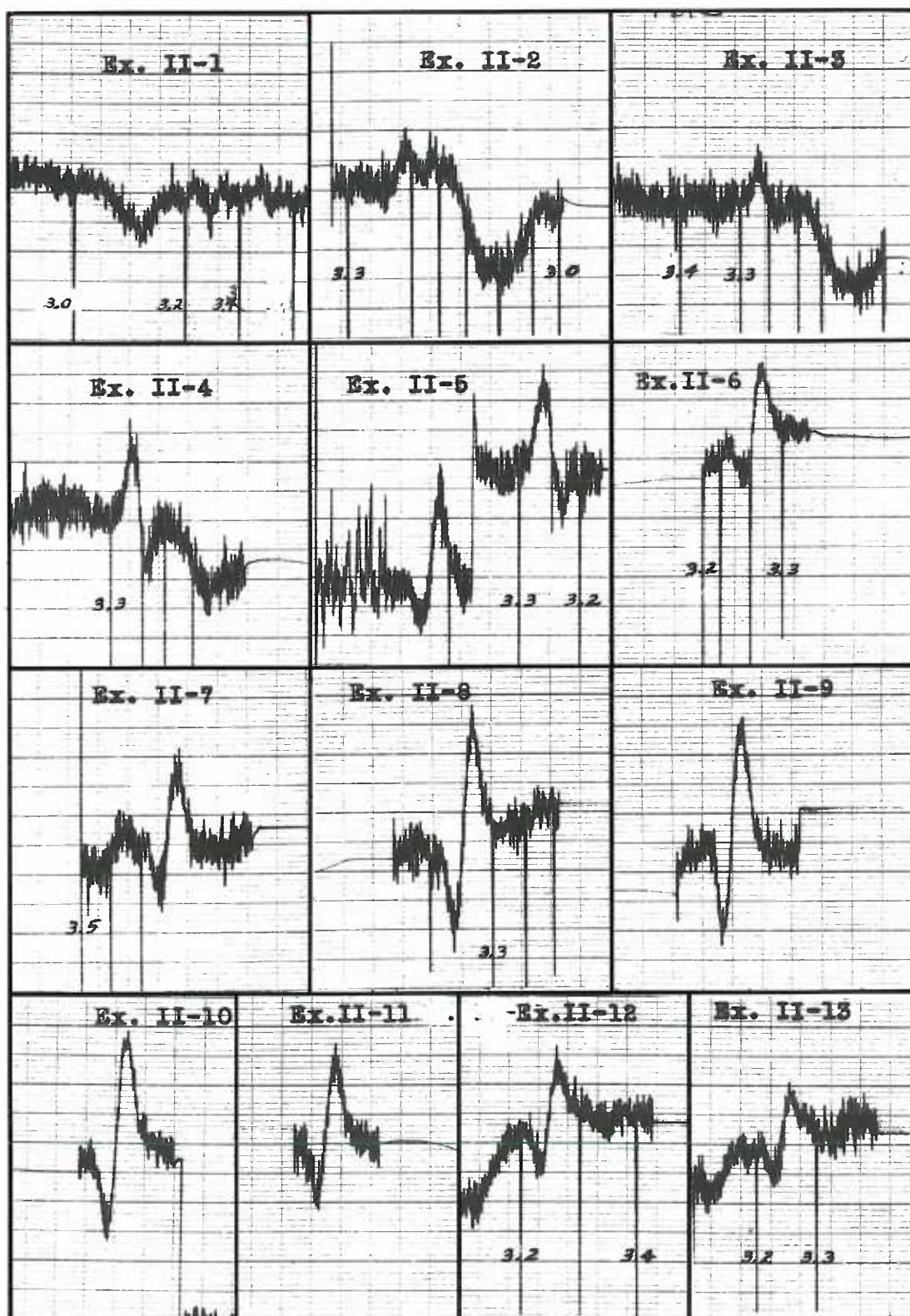enzyme lactic oxidative
decarboxylase with sodium
dithionite.

Fig. 9   79.

80.

Figure 10.　a.　EPR spectrum (1st derivative curve)

of free radical signal

of the 1,1-diphenyl-2-picrylhydrazyl

standard (0.0457mM) at 25°C.

Spectrometer settings:

    gain = 2.5
    scan rate = 110 gauss/min.

b.　First integral of the spectrum

curve shown in a.

c.　EPR spectrum (1st derivative curve) of

free radical signal of the carbon

standard (0.0162mM) at 25°C.

Spectrometer settings:

    gain = 2.5
    scan rate = 110 gauss/min.

d.　First integral of the spectrum

curve shown in c.

Fig. 10

81.

DPPH Standard

0.0457mM

25°C

Gain=25

a

Carbon Standard

0.0112 mM

25°C

Gain=2.5

32

c

1st. Integral of a.

b

1st. Integral of c.

d
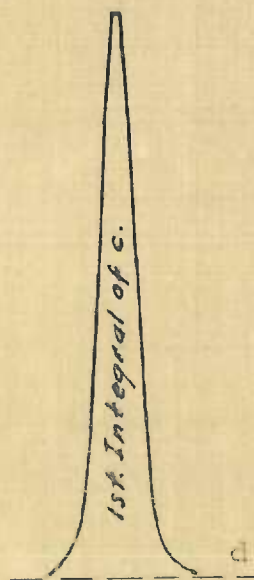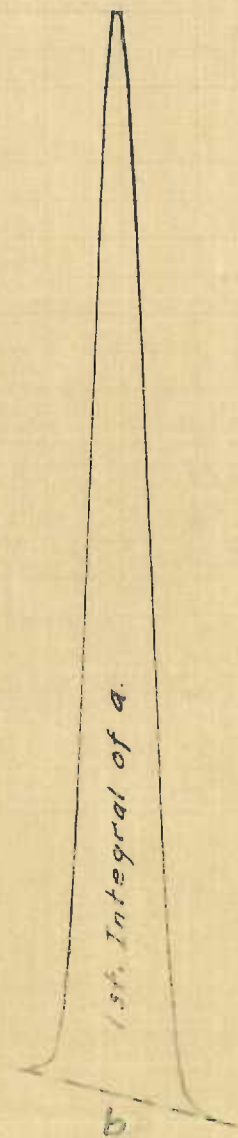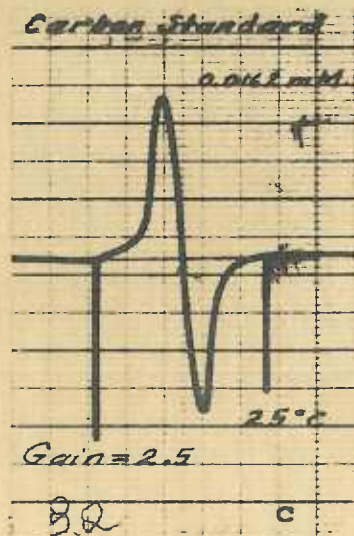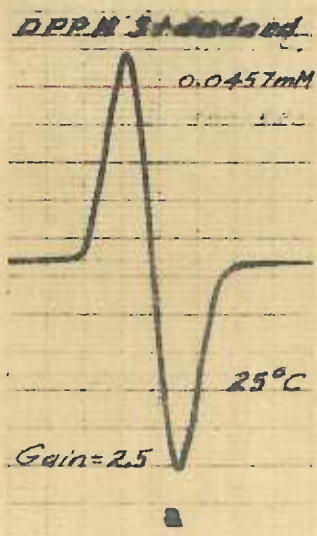
Figure 11. a. EPR spectrum (1st derivative curve)

of free radical signal of

the carbon standard (0.0162mM) at

-138C. Spectrometer settings:

gain = 2.5
scan rate = 264 gauss/min.

b. First integral of the spectrum curve

shown in a.

c. EPR spectrum (1st derivative curve) of

free radical signal of the enzyme at

the critical point (50% reduced),

Experiments II-8, at -138°C.

Spectrometer settings:

gain = 200
scan rate = 264 gauss/min.

d. First integral of the spectrum curve

shown in c.

Fig. 11   83.

Carbon
Standard
0.0162 mM.
Gain = 2.5

-138°c

3.2

3.39   1.

a

L.O.D.
Gain = 200 -138°c

3.2   1.2

Experiment II-8

c
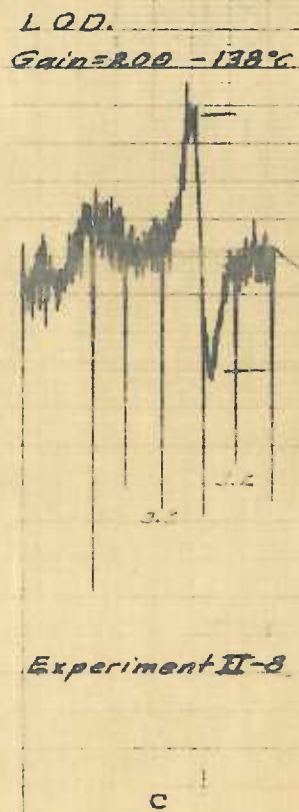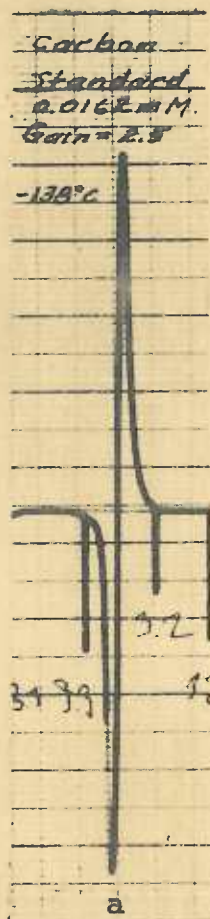
1st Integral of a.

1st Integral of c.

B.   A Free Radical Survey of Some Foodstuffs.

The following tabulation shows the surveyed foodstuffs by category, where free radical was present, the EPR signal height is tabulated, as is a rough approximation of the free radical concentration.   Transition metals observed are also noted:

Category and Item	t°C.	Free Radicals EPR Signal Ht. mm. Gain=100	Approx. FR Conc. mM.	Transition Metals and g-value range	Fig.
BEVERAGES:					
Milk, whole, fresh, pasteurized	-173	55	0.00012	Cu 2.5-2.6	12
Milk powder, fat free	25	5	0.00003		12
Cocoa, dry	25	510	0.00295		12
Coffee, ground, dry	25	172	0.001		13
Tea, dry	25	260	0.0015	1.9-2.2 Mn	13
CEREALS:					
Flour, white unbleached, dry	25				14
Wheat cereal, prepared, dry	25	29	0.00017		14
Wheat cereal, unprepared	25				14
Bun, white	-167				14
Bun, white toasted	25	28	0.00016		14

Category and Item	t°C.	Free Radicals		Transition Metals and g-value range	Fig.
		EPR Signal Ht. mm. Gain=100	Approx. FR Conc. mM.		
Rice, polished dry	20				14
Rice cereal, prepared, dry	20	145	0.00084		14
Coconut, shredded	25			1.9-2.2 Mn	
CONDIMENTS					
Pepper, black	25	180	0.00104	1.9-2.2 Mn?	16
Cinnamon	25	230	0.00133	1.9-2.2 Mn?	17
Paprika	25	21	0.00012	1.9-2.2 Mn 4.2-4.7 Fr?	17
Sage, rubbed	25	62	0.00036	1.9-2.2 Mn?	18
Chili powder	25	36	0.00021	1.9-2.2 Mn? 4.2-4.7 Fe	19
Onion salt	25	4	0.00002	1.9-2.2 Mn?	20
Tomato Ketsup	-173			1.9-2.2 Mn? 4.2-2.2 Fe?	20
MEATS AND FISH					
Hamburger, very well done	21	10	0.00006		21
Ham, baked	-150			Cu	22
Tuna, canned	-150			4.2-4.7 Fe	21
Cheese, cheddar	-150	5	0.00001	2.7-3.2 Cu	22

Category and Item	t°C.	Free Radicals		Transition Metals and g-value range	Fig.
		EPR Signal Ht. mm Gain=100	Approx. FR Conc. mM.		
VEGETABLES					
Carrot, raw	-173	7	0.00001		23
Celery, raw	-173			1.9-2.2 Mn	23
String bean, canned	-149	60	0.00015	1.9-2.2 Mn 9.2-4.7 Fe	15
Potato chips	25	32	0.00019		23
FRUITS					
Apple, raw	-173	17	0.00004		24
Banna, raw	-173	9	0.00002	1.9-2.2 Mn 4.2-4.7 Fe	24
Peach, canned	-173	6	0.00001	1.9-2.2 Mn	24

As state above, the free radical concentrations shown are rough approximations and probably dependable only to the order shown. The sampling was in no way statistical; single samples were selected and run only once.

Figure 12.   EPR Spectra of Milk and Cocoa

Fig. 12   88.

Milk (fresh)

(a)   G=20

Milk (powdered)

(b)   G=80

Cocoa (dry)

FR

(c)   G=10
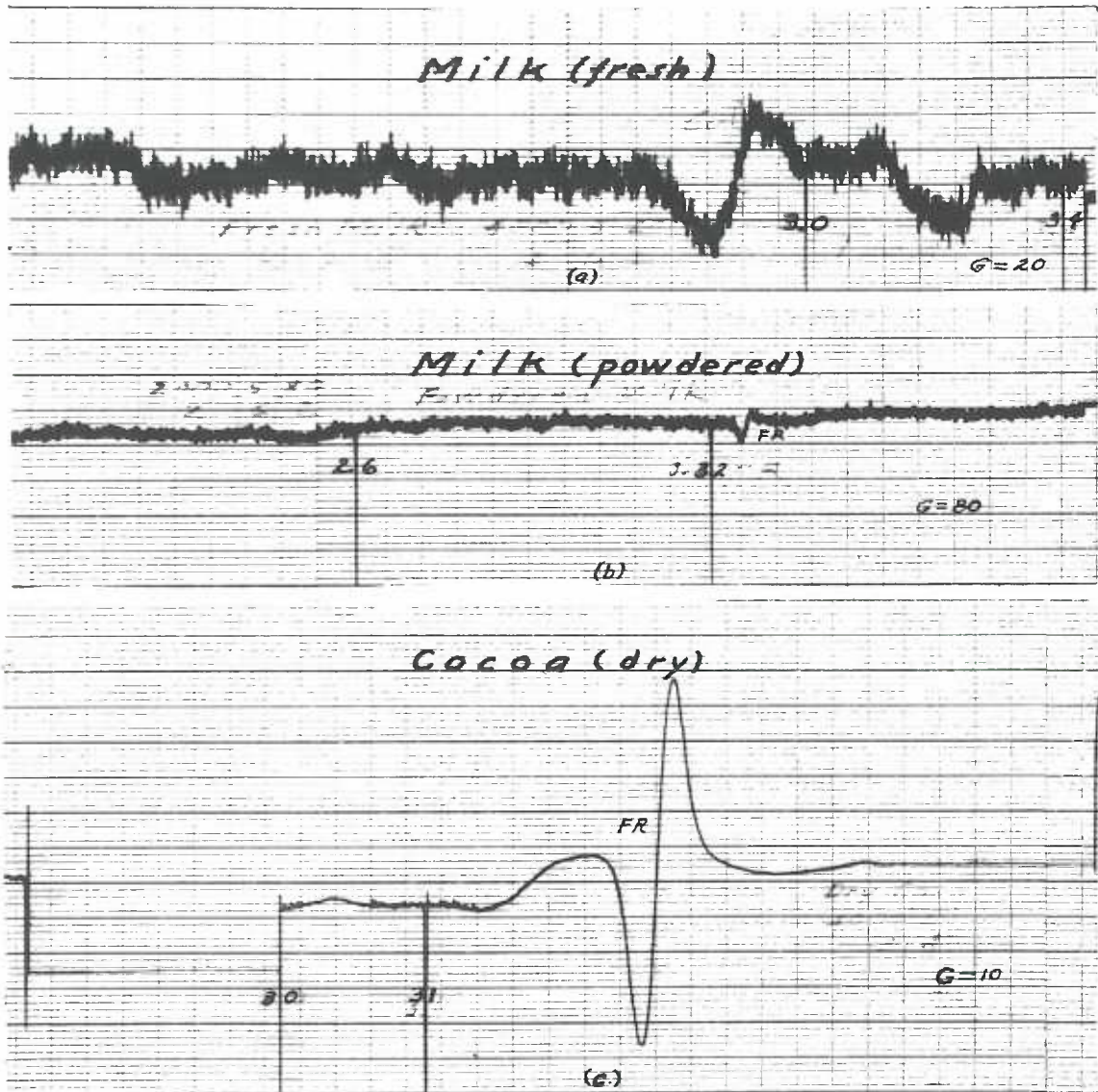
Figure 13.   EPR Spectra of Coffee and Tea

Fig. 13.   90.

Coffee.

Gain=50

Tea.

Gain=10
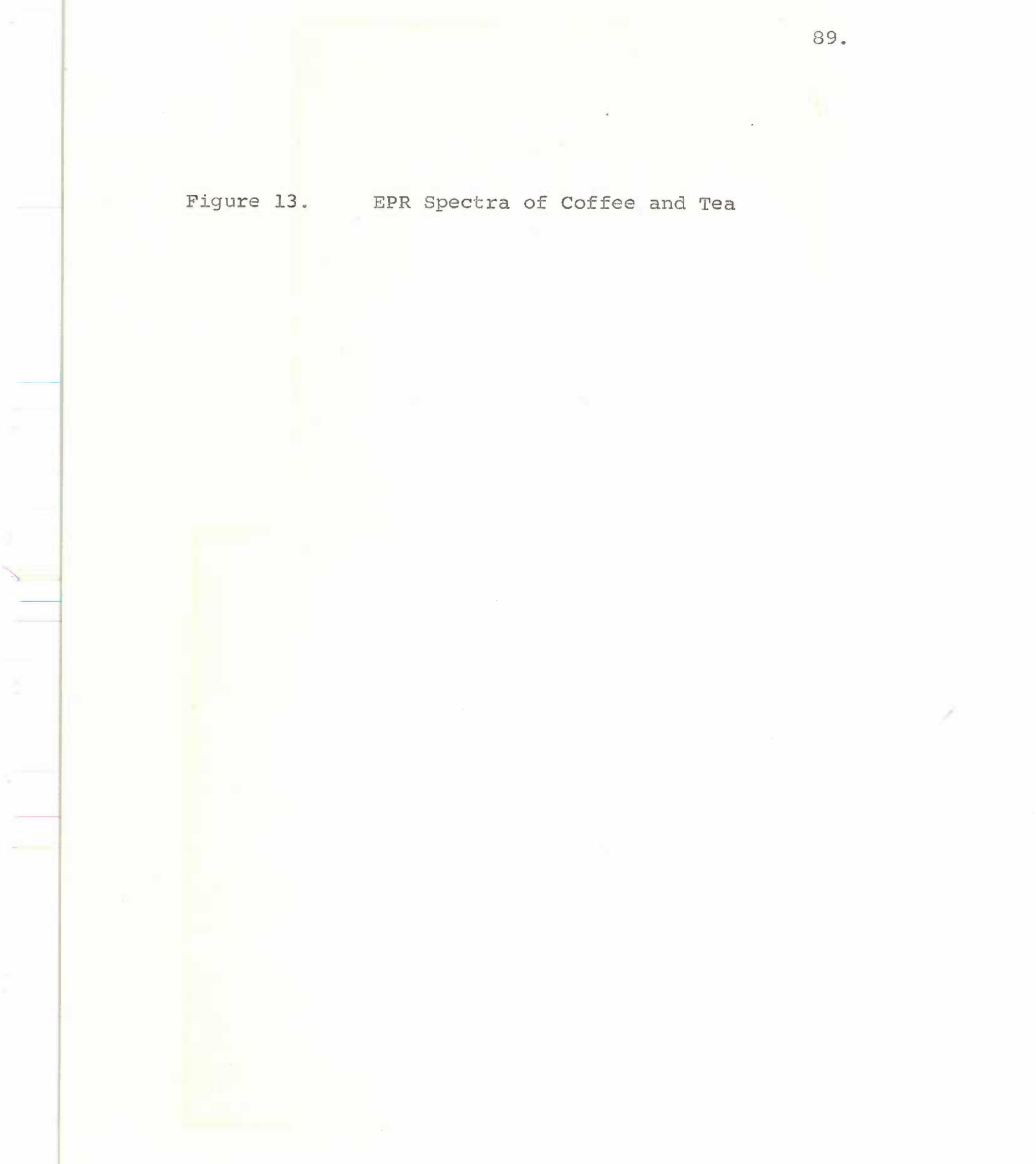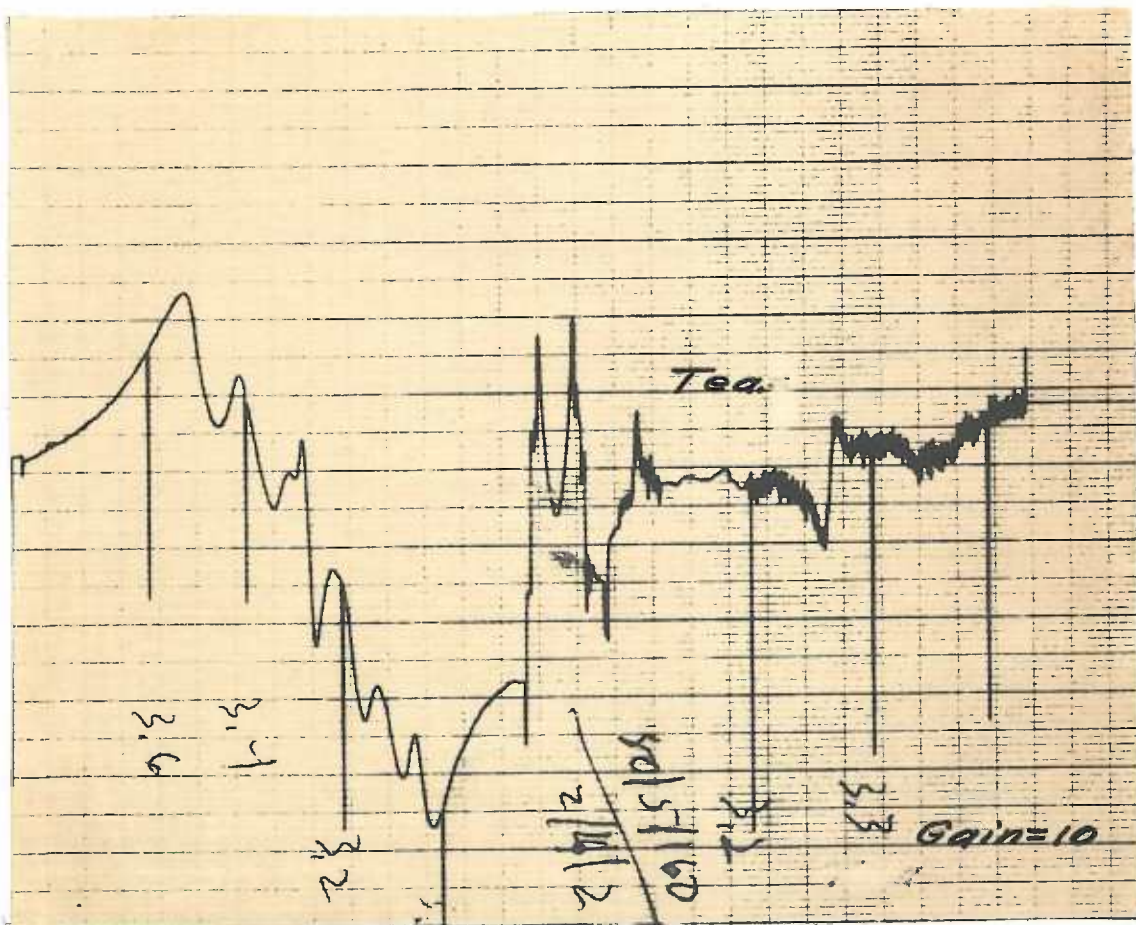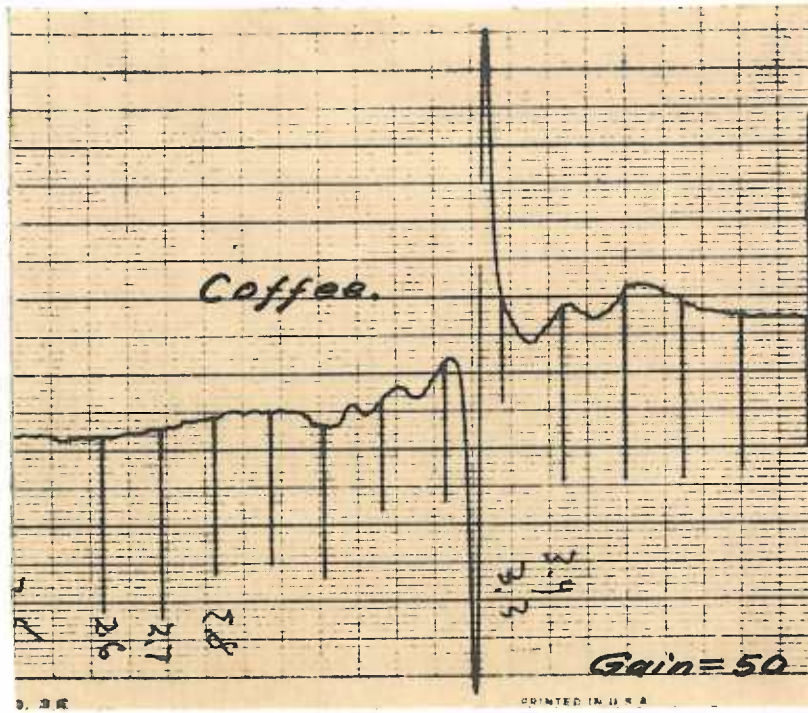
Figure 14.   EPR Spectra of Cereals.

Fig. 14

Flour
white, unbleached

G=100

3.0   3.2

(a)

Puffed Wheat

FR

G=100

3.0   3.2

(b)

White Bun
Untoasted

Cu

3.2

G=100

(c)

White Bun
Toasted

FR

3.0   3.2

G=100

(d)

Wheat Cereal
Unprepared

TM

3.4

3.0   G=100

(e)

Rice Cereal
Prepared

FR

G=20

(f)

Rice

TM
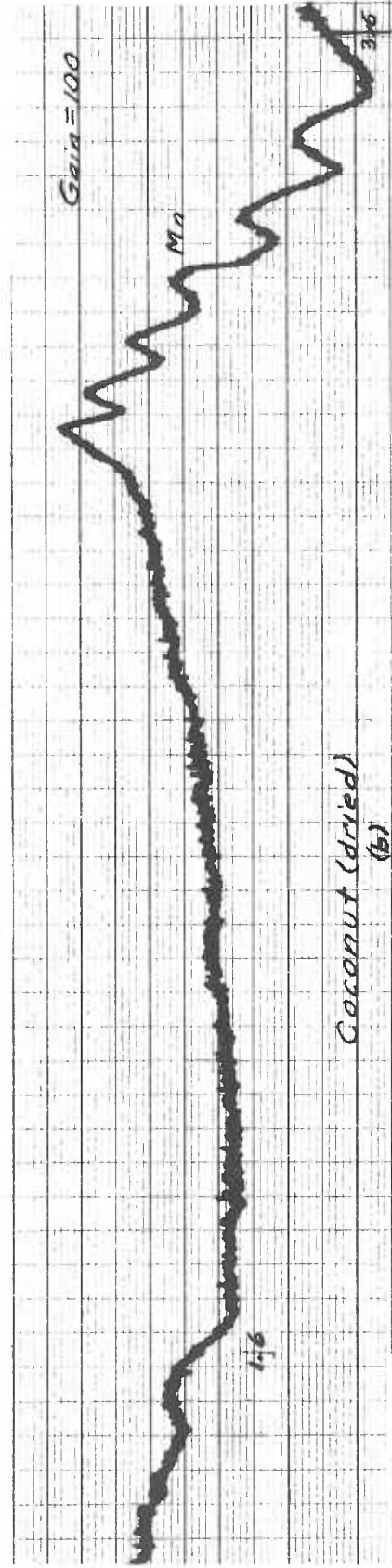
3.7

3.0   (g)   G=100
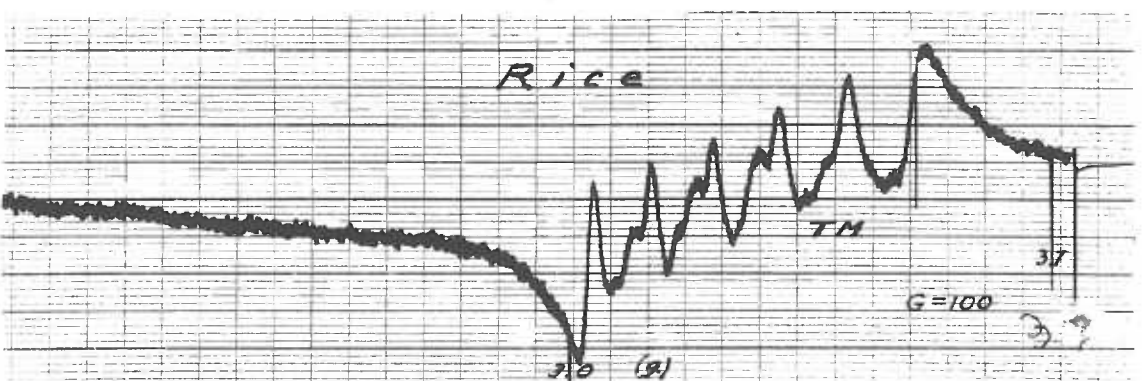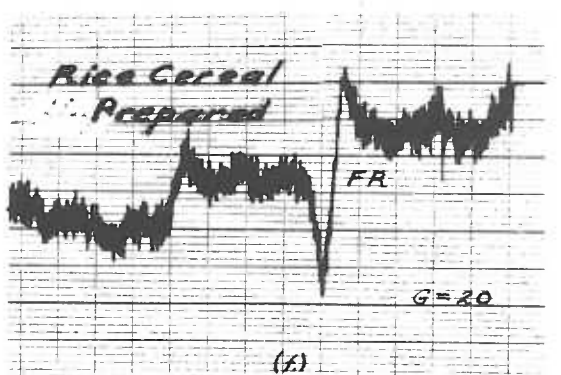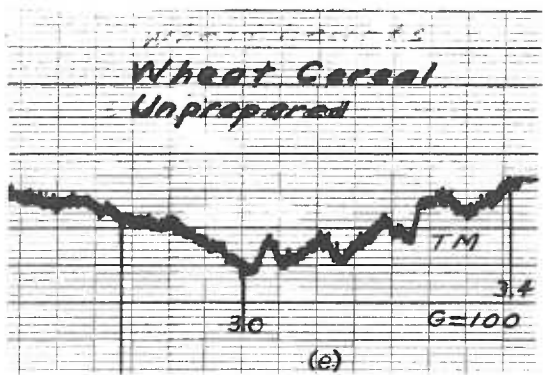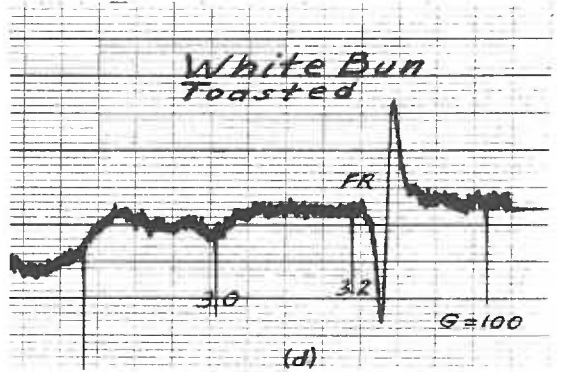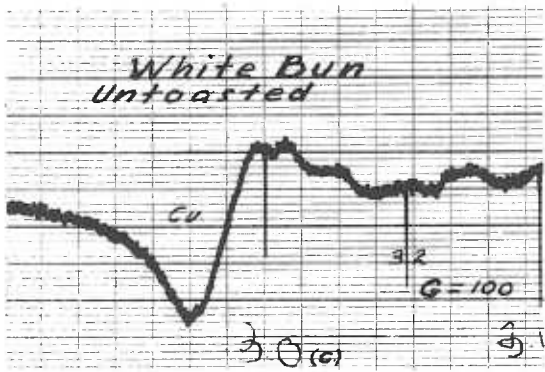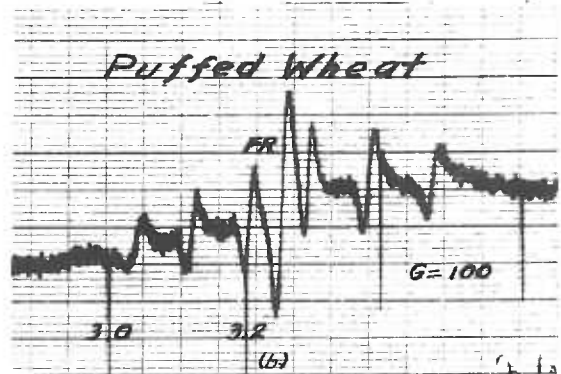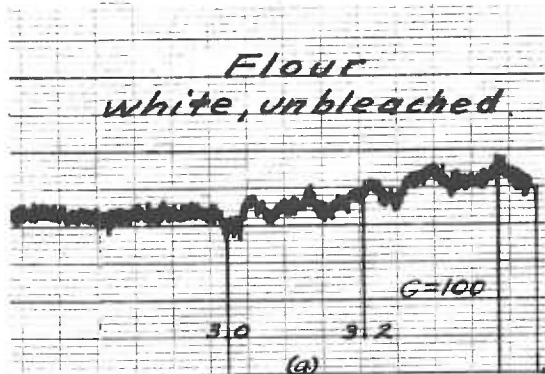
Figure 15.   EPR Spectra of String Bean

and Coconut

Fig. 15

String Beans (canned)
(a)

Coconut (dried)
(b)

Figure 16.   EPR Spectrum of Black Pepper

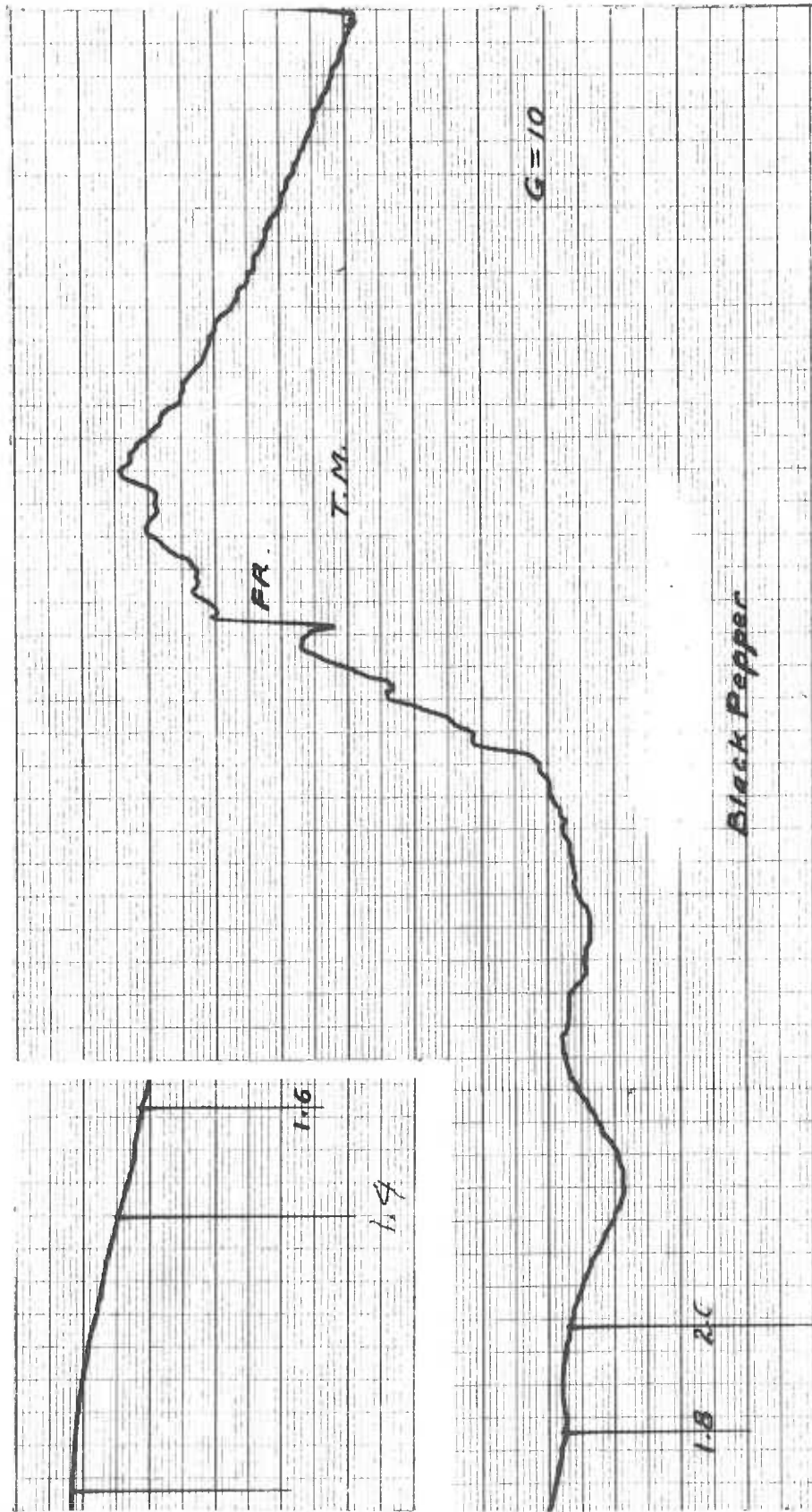Fig. 16   96.

Black Pepper

Figure 17.   EPR Spectra of Paprika and Cinnamon

Fig. 17   98.

Fig. 17
(a) Paprika
(b) Cinnamon

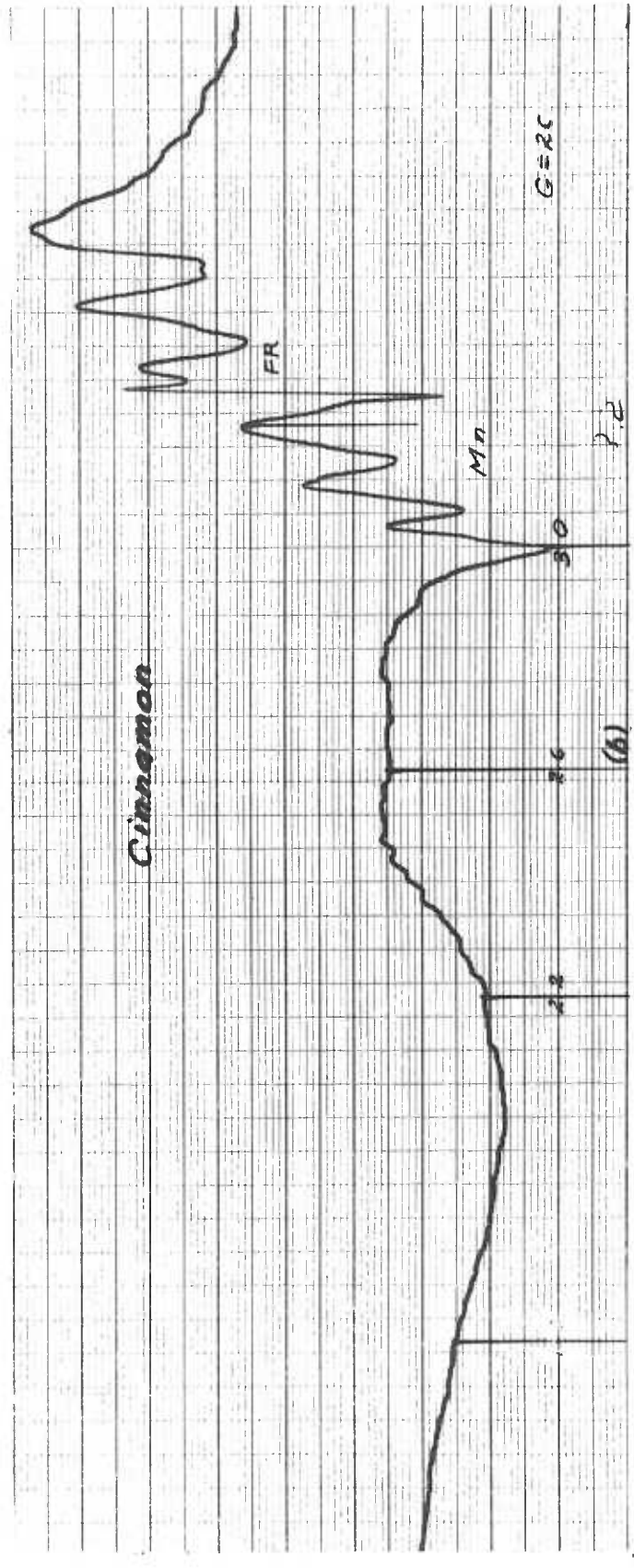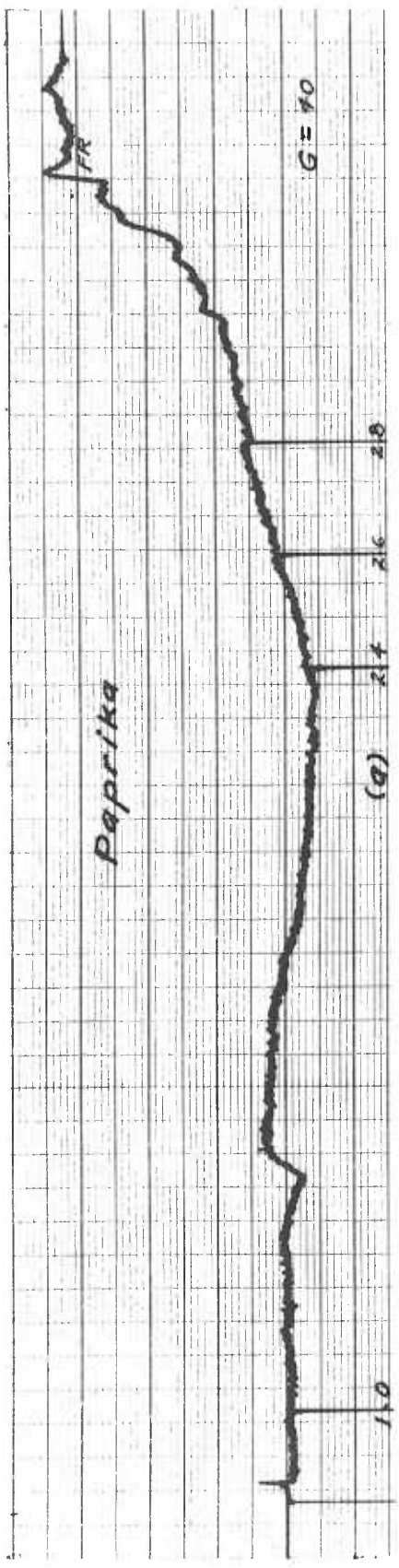Figure 18.   EPR Spectrum of Sage

Fig. 18.

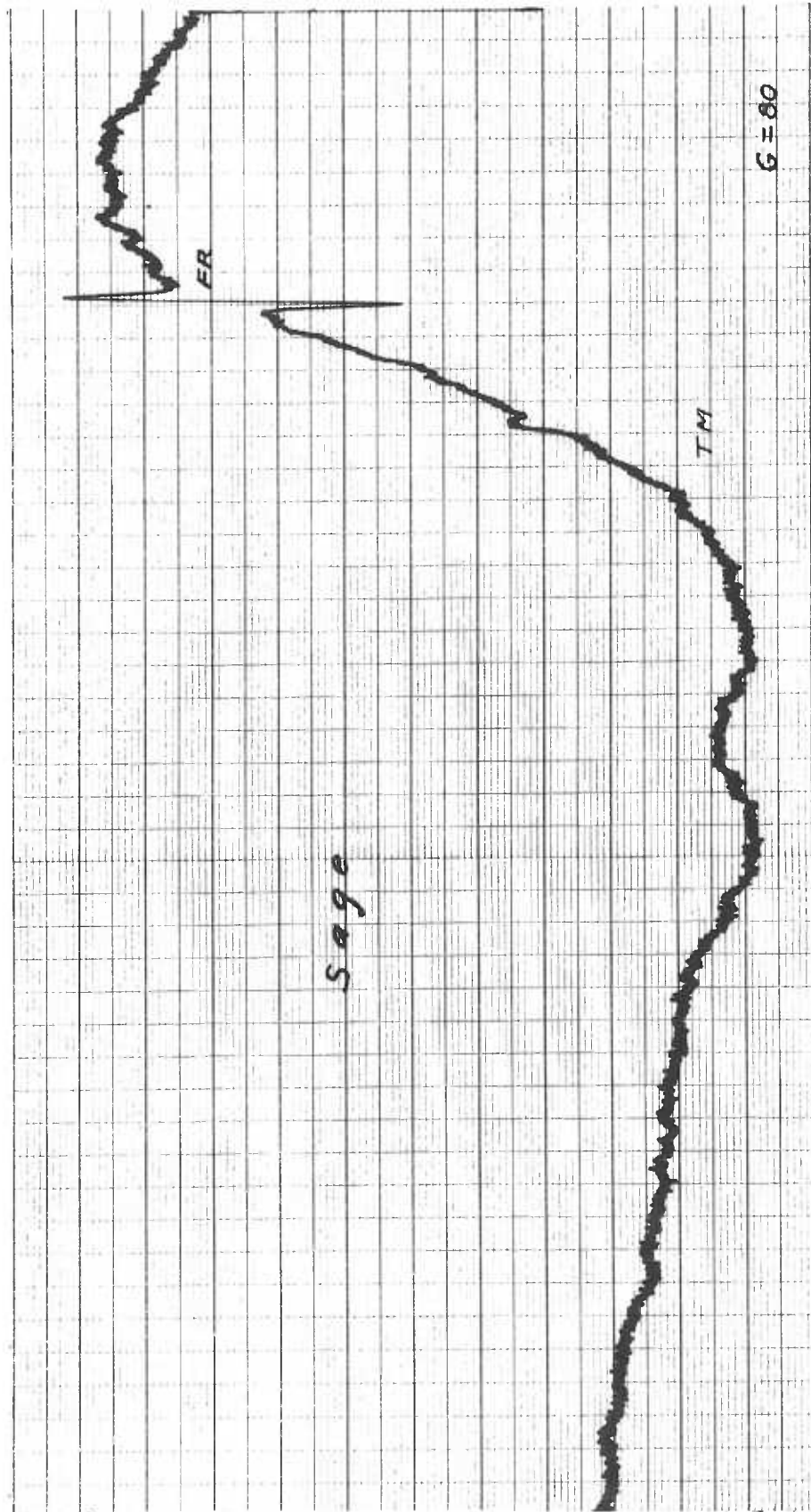Fig. 18.   100.

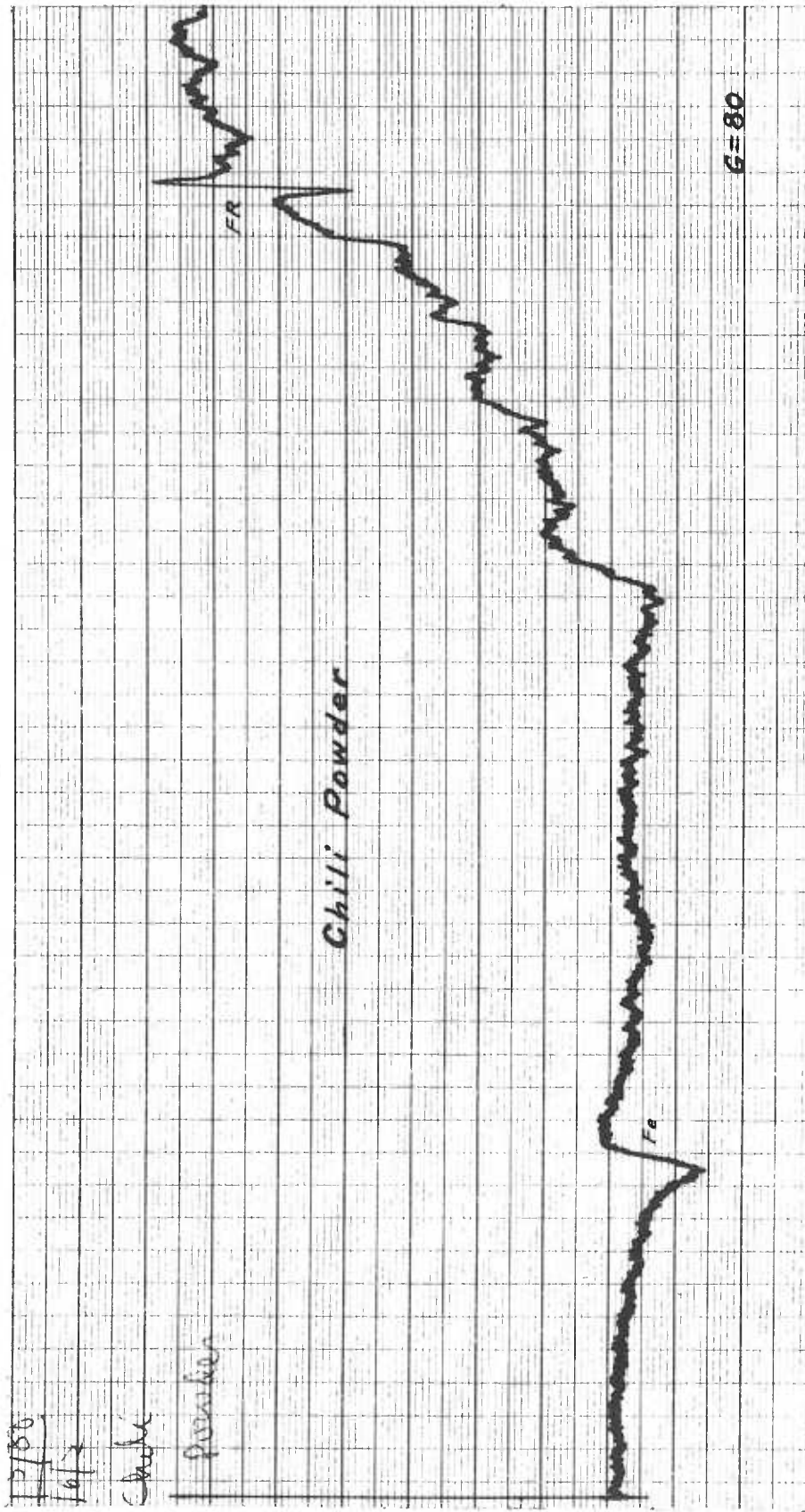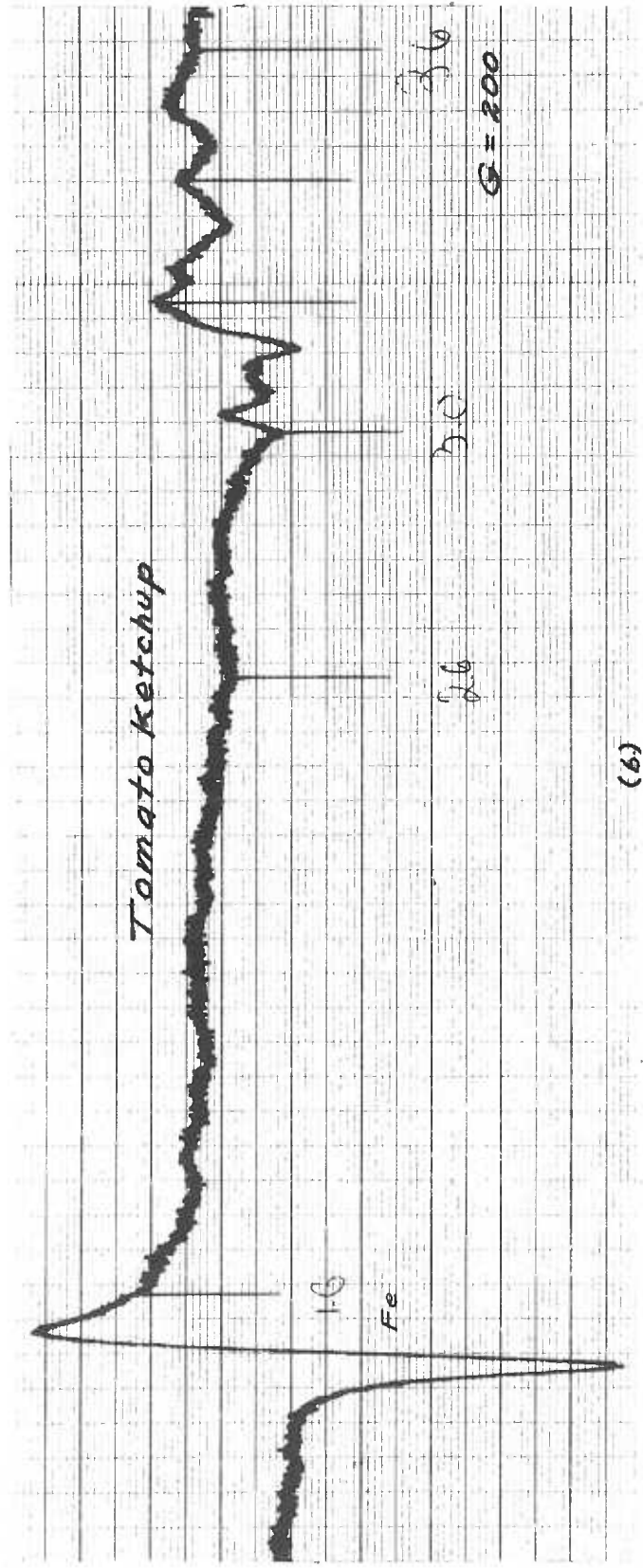Figure 19.   EPR Spectrum of Chili Powder

Fig. 19

Chili Powder

Figure 20.   EPR Spectra of Onion Salt and
              Tomato Ketchup

Fig. 20

Onion Salt

(a)

Tomato Ketchup

(b)
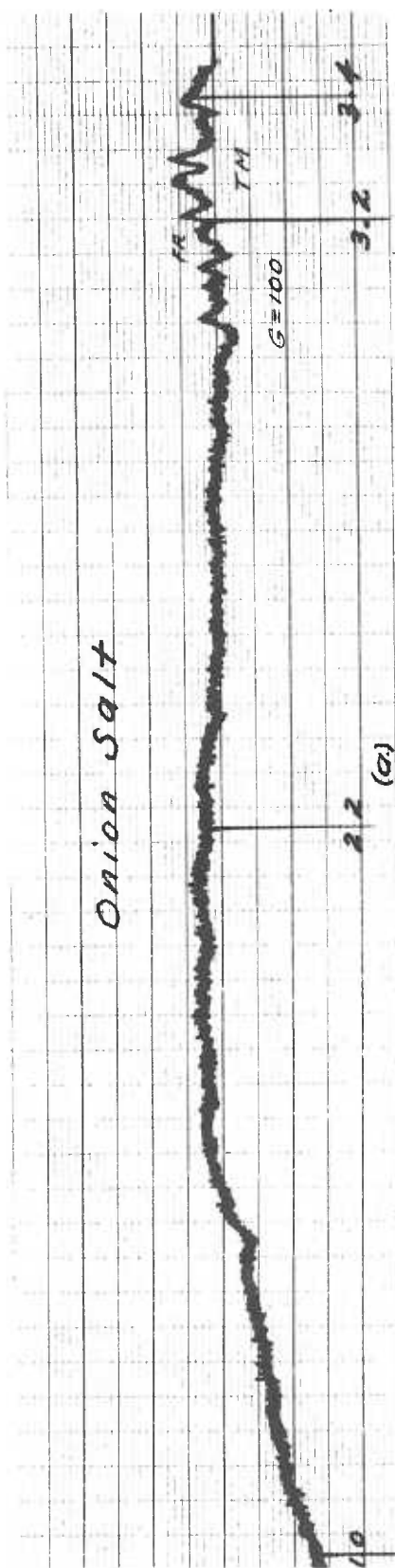
Figure 21.   EPR Spectra of Hamburger and Tuna

Fig. 21   106.

(a) Hamburger (well done)   G = 100

(b) Tuna (canned)   G = 100
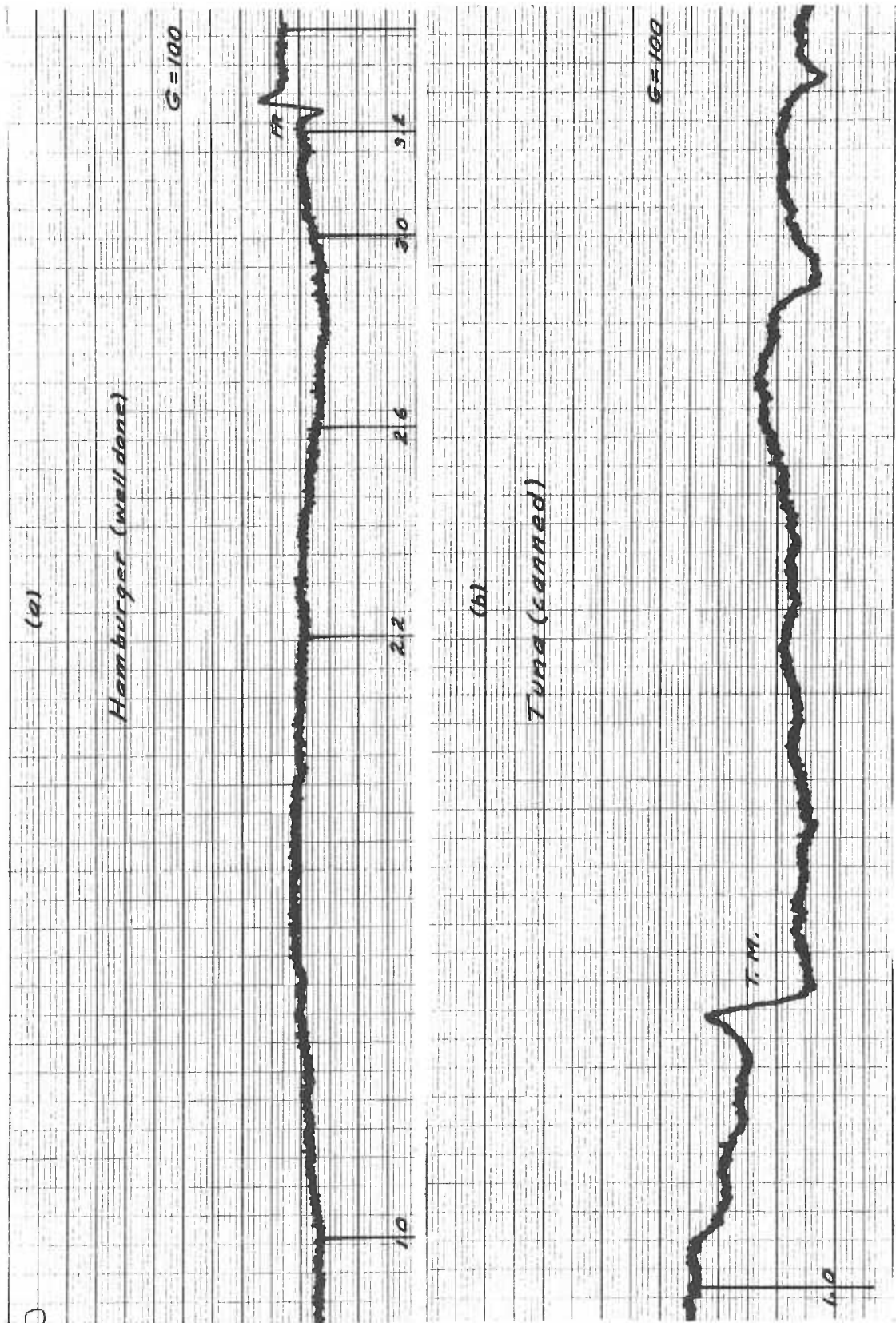
Figure 22.   EPR Spectra of Baked Ham and Cheddar Cheese

Fig. 22

G = 100

3.7

T.M

Cheddar Cheese

(b)
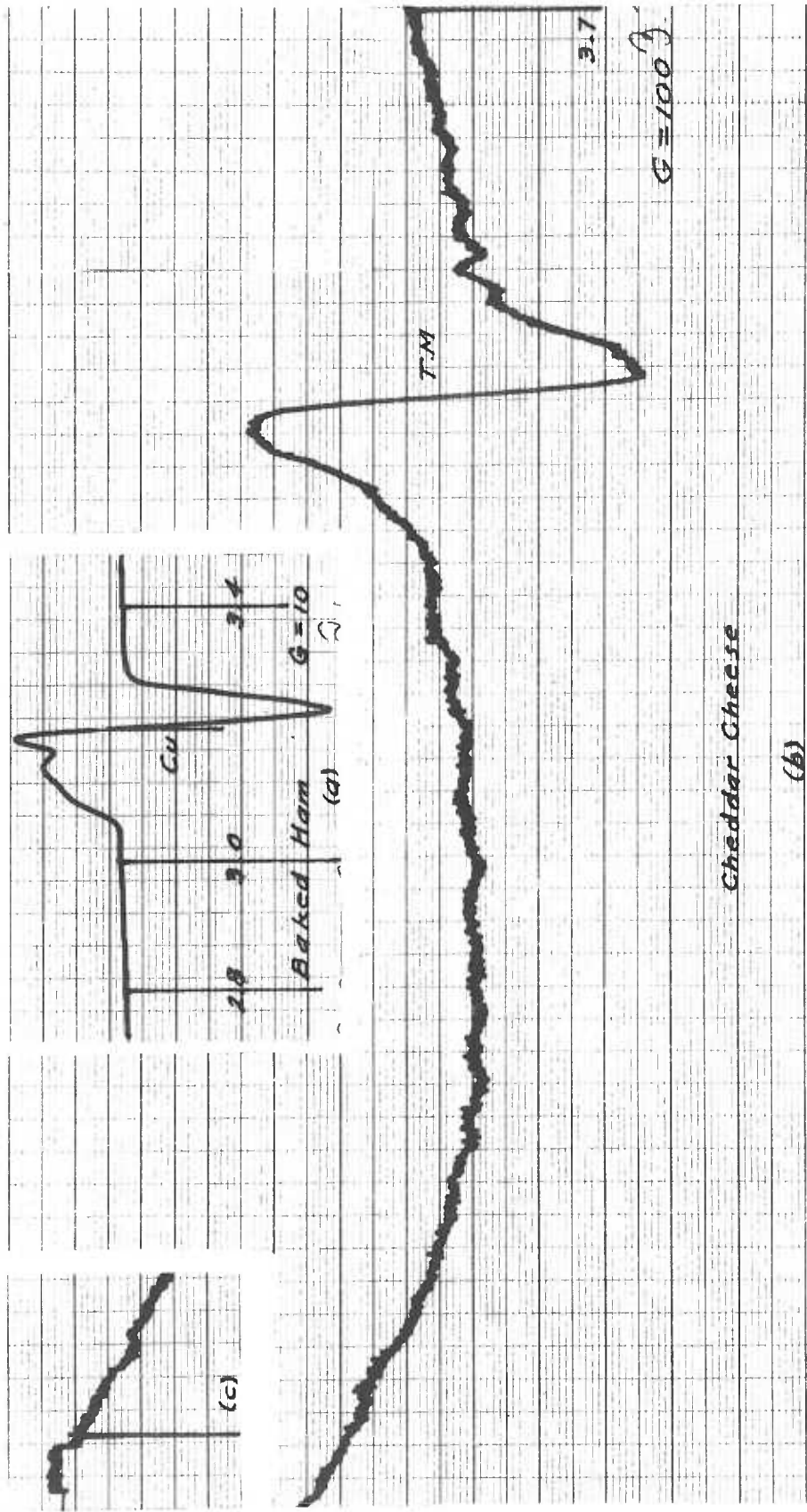
Baked Ham

(a)

2.8   3.0   Cu   3.4

G = 10

(c)

Figure 23.   EPR Spectra of Potato Chips,
                Celery and Carrot

Fig. 23.

Potato Chips
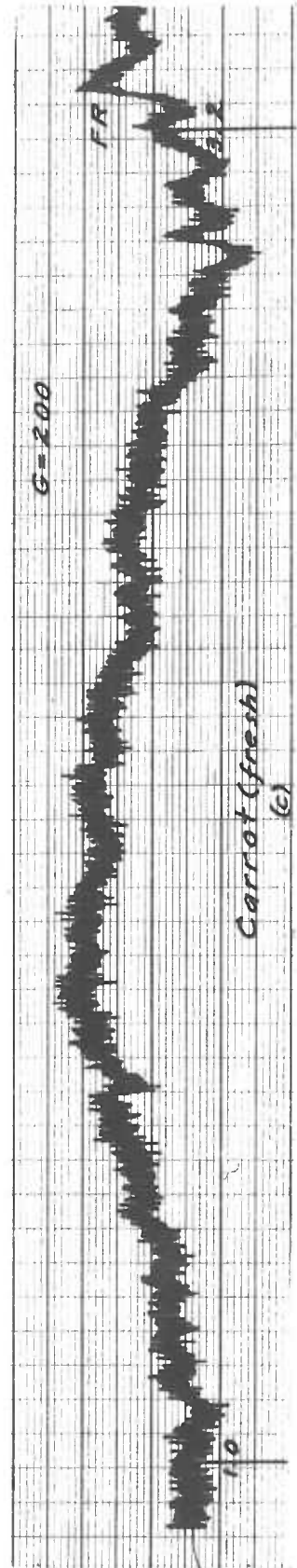(a)

Celery (fresh)
(b)
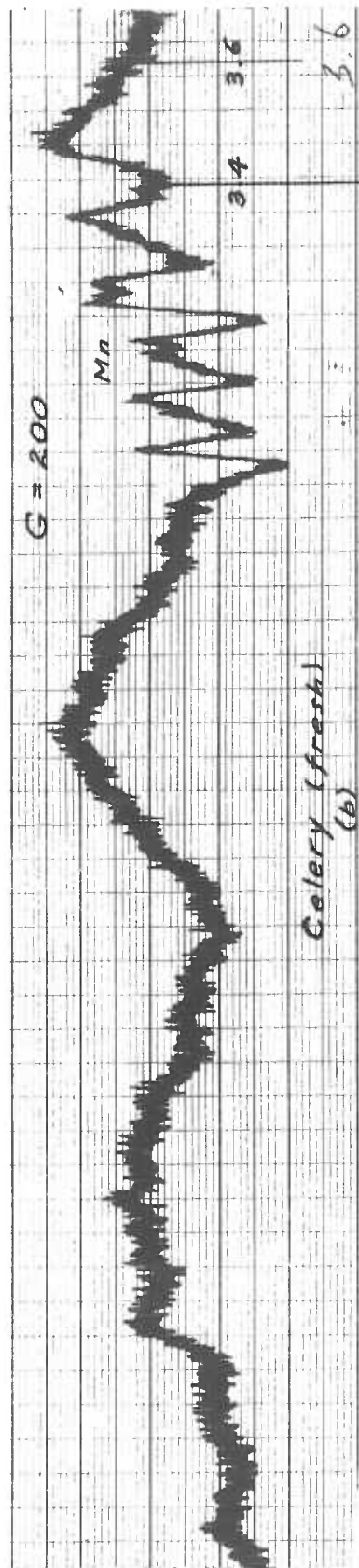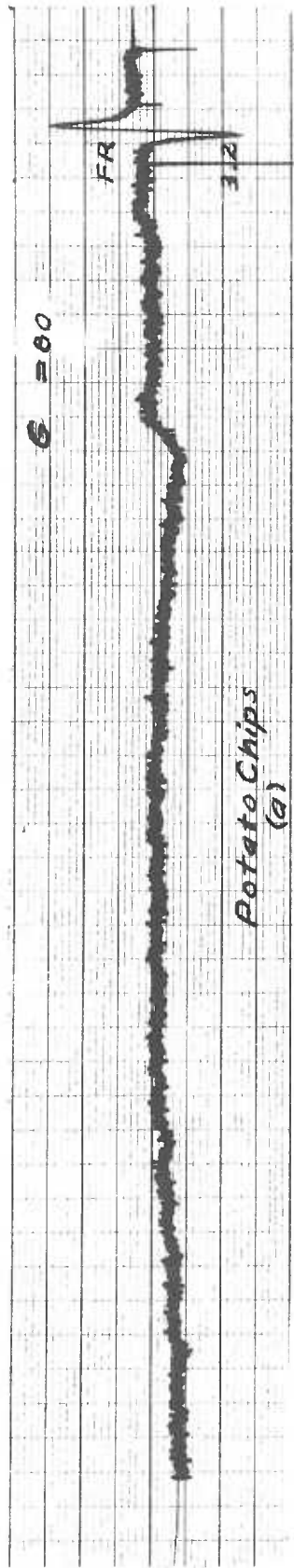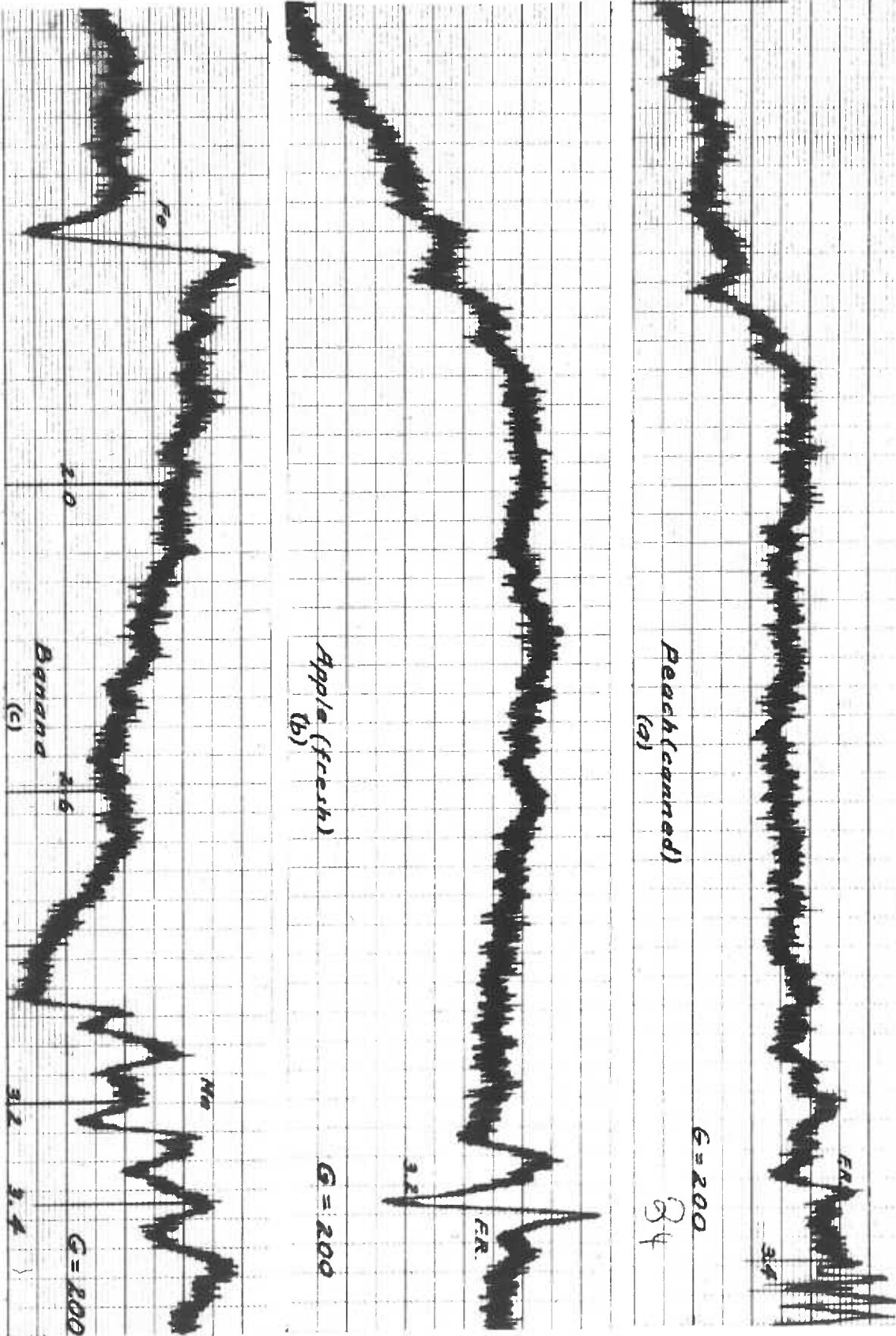
Carrot (fresh)
(c)

Figure 24.   EPR Spectra of Peach, Apple and

Banana

Fig. 24.

Fig. 24.

IV DISCUSSION

A. Determination of A Free Radical Formation Constant

The primary purpose of the work herein described has not been the establishment of an exact quantitative constant, although it is hoped that such has been achieved, but rather a study of method and technique in order to determine their applicability to this type of investigation.

The outcome indicates that the method of anaerobic titration with EPR spectrometer monitoring is easy to use and gives highly reproducible results.

The major instrument, the EPR spectrometer, is of course extremely costly and not now available at many locations, but such instruments will perhaps be considerably more plentiful at a later date.

With respect to the results obtained, there is little more to say than has already been stated in the "Results" section.

At the present time it is impracticable, and in fact very nearly impossible, to duplicate the exact anaerobic condition from one experiment to the next, and this situation makes impossible correlation of the quantities of reducing agent used in different experiments. However, as pointed out under "Results", the critical point is found at the

same location regardless of variation in the anaerobic condition from one titration to another. Nevertheless, it is quite essential to maintain the same anaerobic condition throughout any one experiment (titration), in order to detect mechanisms that are active at any time during a complete titration.

Such a mechanism seems to occur in the early stages of the anaerobic titration of lactic oxidative decarboxylase and can be seen as a reverse curve in the ascending slopes of the titration curves of Experiments II and III, Fig. 6. This reversal of curvature on the ascending slopes also occurs during the titration of FMN and a senior investigator has recently suggested that it signals the formation of a charge transfer complex.

The work herein described also demonstrates that extremely minute quantities of material can be handled by using a gas tight syringe, micrometer drive and capillary delivery tip. It also demonstrates that anaerobic processes can be employed with maximum convenience without relatively elaborate apparatus, such as a glove box.

B. A Free Radical Survey Of Some Foodstuffs

This study as stated under results was in no way statistical and was strictly in the nature of a reconnaissance survey.

Immediately apparent is the presence of free radicals in foods exposed to rather high temperatures, e.g. toasted buns, prepared cereal, well done hamburger, canned fruits and vegetables and potato chips.

The condiments also in general seem to have a fairly high free radical content.

Cocoa is especially rich in free radicals.

The transition metal content of foods has been noted as a matter of interest since they show on the EPR spectra.

There is no attempt made at this time to attach any significance to the particular free radical content of various foods. Furthermore, no suggestions of correlations between this value and effect on health is intended, at this time.

V SUMMARY AND CONCLUSIONS

Summary

There has been presented here: (1) a brief general discussion of oxidation and reduction, with emphasis on biochemical redox mechanisms, along with pertinent historical background, (2) a brief discussion of free radicals, and their occurrence, detection, and quantitative measurement, (3) review of knowledge about the enzyme, lactic oxidative decarboxylase, (4) description of a method for the anaerobic, reductive titration of oxidative enzymes, with EPR monitoring of the resultant free radical levels; and application of the method to the enzyme lactic oxidative decarboxylase; and use of the data obtained to calculate the free radical formation constant.

Also presented are the results of a survey of a few selected foodstuffs for the purpose of determining their free radical content.

Conclusions:

1. Anerobic titration of oxidative enzymes, with EPR monitoring of the resultant free radical levels is a convenient method and gives reproducible results.

2. This method was applied to the enzyme lactic oxidative decarboxylase titrated with sodium dithionite; the quantity of free radicals was determined at the equilibrium point

and the data was used to calculate the free radical formation constant as 6.72×10^{-7}.

3. Many of the foodstuffs surveyed were found to contain free radicals, especially those processed at fairly high temperatures, and condiments.

VI ABREVIATIONS

DPPH   is   1,1- diphenyl-2-picrylhydrazyl

EPR   is   electron paramagnetic resonance

FMN   is   riboflavin-5-phosphate

FR   is   free radical

LOD   is   lactic oxidative decarboxylase

Q   is   quinone

QH   is   hydroquinone

SQ   is   semiquinone

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