A study of the catecholase activity of the enzyme tyrosinase : a thesis

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INTRODUCTION

The term "oxidation" has been used for the process of combustion from the time of Lavoisier. The liberation of energy by this process has been known from ancient times. The mechanism whereby oxidation leads to the release of useful energy is still unknown. Living things, as highly complex ordered systems, require a constant energy input for their maintenance. This they derive from the universe by an intricate mechanism of step-wise oxidation. They exist between the order of the cosmos which is giving off energy of disintegration and the chaos of oblivion, in which the energy of the system is degraded to maximum entropy.

Life on this earth derives virtually all energy from the sun by radiation. Chlorophyll-containing plants trap the radiated energy and convert the products of combustion, carbon dioxide and water, back into organic compounds which can be used by other living things. Slowly, by involved and intricate mechanisms, plants and animals bring about the recombination of these organic compounds with oxygen to give again carbon dioxide and water, completing the cycle and extracting the power of the sun for the functions of life.

One of the central problems of modern biochemistry is the mechanism of biological oxidation. In order to study the systems which catalyse biological electron

transfer in tissues, these systems are isolated and examined as molecular processes. One such system is the widely distributed enzyme, tyrosinase, and its substrates. It is the study of the mechanism of tyrosinase action which is the subject of this thesis.

The enzyme tyrosinase has been studied for eighty-five years, but the mechanism of its action is still obscure. Although Kubowitz (39) proved that it contains copper in the active site, the role of the copper in the activity has not been elucidated.

Tyrosinase has some features which are unique among enzymes. It has a dual activity; it suffers a high degree of reaction inactivation; it is not blue even though it contains copper. The purpose of the research reported herein is to add a little bit to the accumulating data concerning this enzyme so that its nature and function in the life process may eventually be understood.

OXIDATION AND REDUCTION

Oxidation has been defined by Michaelis (55) as the removal of electrons from a substance and reduction as the receipt of these electrons by another substance. These processes always occur concurrently, and the systems in which they occur are often designated as redox systems. In wholly inorganic systems such as $Fe^{++} \rightarrow Fe^{+++}$, in the presence of an oxidizing agent which is thereby reduced, it is usually easy to follow the overall path of electron transfer, but not at all easy to explain how or why it takes place. Simple inorganic reactions are often far more complex than their usual chemical equations would indicate. (71)

The tendencies of various elements to participate in oxidation-reduction reactions have been measured by means of the potentials established when the pure element is in contact with a solution of its ions at an activity of one. Hydrogen at one atmosphere in contact with hydrogen ions at an activity of one has been selected as the standard with an arbitrary potential of 0. Standard potentials for the common elements are listed in tables which are now an essential part of most elementary chemistry textbooks.

Organic compounds can also exist in various oxidation states, although we generally think of the oxidized form as being different compound from the reduced form, e.g. methylene blue and its leucobase.

These materials can be listed also in order of the potentials established when the oxidation form is in contact with the reduced form (8).

Redox potentials are a measure of the "electron pressure" existing in the reduced form of a material with respect to the oxidized form. No actual electron transfer can take place as long as energy barriers exist which prevent the physical movement of these electrons from the compound in which the escaping tendency is greater to that in which it is less. These barriers may be distance, an impermeable container, a very high energy intermediate compound such as a free radical, the repulsive force of like charges, molecular shape, or any other force which tends to absorb, block or divert electrons. These energy barriers may be reduced by various means, such as: (1) mixing solutions of two reactants together to overcome the barriers of distance and impermeable walls, (2) by adding a catalyst to absorb the reactants, bringing them closer together physically, or changing one or both to a more active form, e.g. in hydrogenation by the use of Raney nickel or platinum, (3) by raising the energy of the reactant compounds high enough to overcome an energy barrier, e.g. a 10° rise in temperature approximately doubles the rate of chemical reactions, (4) by addition of salts, surface-active agents, electrical discharges, or special catalysts, or selection of solvent to reduce the

repulsion of like charges, as in the precipitation of colloids from solution, (5) by selection of a special catalyst which alters the steric configuration of reactants so that reaction takes place more readily. Probably most enzymes are catalysts of the last type but also partake of the nature of one or more of the other types.

A reaction goes spontaneously if a thermodynamic pressure (negative free energy difference) exists in a system, provided any energy barriers which also exist in the system are overcome by one or more of the means mentioned above. The simple example of the production of ammonia by the Haber process illustrates the principle involved. Since nitrogen and hydrogen do not react together at room temperature at a practical rate for ammonia production, a catalyst is used along with elevated temperature to start the reaction. It then proceeds spontaneously until the "electron pressure" difference between reactants and products no longer exists. The system is then said to be at equilibrium. The reaction does continue to take place, but the reverse reaction, which also begins to take place as soon as products exist to react together, goes at the same speed at equilibrium and there is no further change in the components of the system. The energy of the system is a part of the reaction, and a reaction which would go one way spontaneously because of the release

of free energy can be forced in the opposite direction by allowing it to take place in an energy-rich environment. The ammonia synthesis is actually forced in a direction unfavorable to the production of ammonia by increased temperature, although the heat activation is necessary to get the reaction started. This is true of all "exergonic" reactions, according to the principle of Le Chatelier.

BIOLOGICAL OXIDATION AND REDUCTION

The above principles apply to the more complex biological systems to be discussed below as well as to the well-known examples in inorganic chemistry mentioned. The interrelationships between these large and complex molecules are more complex and some problems of structure and reactivity apply only to these larger aggregates. Proteins and polysaccharides are colloids and show phenomena of absorption, light scattering, etc. peculiar to colloid structure.

Tables of the more common complex organic materials which make up biological redox systems have been compiled (16, pp. 412-413) and have been very useful in helping to establish the steps in the terminal respiratory chain of mammals. The cytochromes studied first by Keilin (35) have been shown to participate in the transfer of electrons from the food materials ingested by the animal to oxygen. The order in which these

cytochromes receive and pass along electrons has been deduced partly from redox potentials and partly from *in vitro* studies. By no means all the problems are solved in this field, however.

When Michaelis (55) considered the problem of biological electron transfer, it occurred to him that most organic redox reactions take place with the overall transfer of only one or two electrons at the most. It seemed to him more probable that the electrons are transferred one at a time. "Electrons are transferred from one molecule to another singly, and any bivalent oxidation or reduction can be resolved into successive univalent steps." The second electron could follow more or less quickly, even overlapping the transfer of the first, provided the kinetic energy of the molecules is sufficient to overcome the activation energy and the total transfer is within the potential range of the oxidizing agent. The reaction would then appear as a two-electron transfer, or even more, except that it is not usual for the energy of the system to be capable of transfer of more than two electrons. If the second electron followed slowly enough it should be possible to isolate or obtain evidence of the presence of free radical intermediates in these reactions.

Free radicals were first positively identified by Gomberg and then later by Paneth who generated them by heating tetraethyl lead at very low pressure in a stream

of hydrogen and proved that these very reactive molecules would remove metallic mirrors from a tube through which they passed. He correctly deduced that they were the ethyl (and methyl) radicals which were postulated by Bunsen, Gay-Lussac and other early workers by analogy between methyl and ethyl chlorides and the chlorides of metals. (They thought that if metals could be released from their salts and exist in the free state it should be possible to do the same with methyl and ethyl.)

The reactivity of free radicals can reasonably be ascribed to the existence of at least one unpaired electron in their molecules. This, by its very nature, is an unsatisfied valence bond. Certain special physical properties conferred upon a molecule by virtue of the existence of an unpaired electron within it will be considered later.

Michaelis (55) then considered free radicals to be normal constituents of biological redox systems. If he were right, it would be necessary to take into account the peculiar reactions of free radicals in the study of these systems. He thought free radicals more likely to be set free in the reaction medium if electron transfer takes place upon collision of donor with acceptor without formation of an intermediate compound. The chances of termolecular collision are so small as to make reactions involving non-equivalent transfer

extremely slow.

In enzyme-catalyzed redox reactions fairly stable intermediate compounds are nearly always formed. Michaelis (55) gives the example of the hydrolysis of sucrose in which an enzyme-sucrose-water complex is postulated. He states that often ternary complexes involving both donor and acceptor groups with the enzyme are formed. The transfer of electrons in these cases is intermolecular. The possibility of reaction of binary complexes of enzyme and substrate with co-enzyme or electron acceptor is not ruled out, but the difference between this mechanism and the ternary complex is not great. Since enzyme-catalyzed reactions are reversible, the direction of overall reaction is determined by the disappearance of product by further reaction with some other system.

The more recent views of Westheimer (71) take into account the fact that not even the simplest inorganic ions exist in aqueous solution without a shell of coordinated solvent molecules around them. These solvent molecules are oriented in thermodynamically stable arrangements for the particular ions which attract them (71). A part of the stabilizing force which determines these arrangements is the charge on the ions. When the charge is changed different arrangements of solvent molecules may become more stable. The energy involved in reorienting these molecules accounts for a

part of the activation energy of the reaction in which the charge is changed. Reorientation of solvent molecules may also explain why some supposedly very simple redox reactions between inorganic ions take place very slowly, for example, the reaction #

$$Co^{*}(en)_3^{++} + Co(en)_3^{+++} \longrightarrow Co^{*}(en)_3^{+++} + Co(en)_2^{++}$$

Many inorganic redox reactions and most organic ones are thought to take place through a bridged-complex intermediate. Instead of being directly shifted from one ionic or molecular nucleus to another, the electron may be passed through a conducting or semi-conducting system from one end of the complex to the other. The acceptance of the molecular orbital theory makes this theory very plausible. Electrons in pi-type molecular orbitals are delocalized, having more mobility than expected from the fixed direction of a sigma-type bond. Systems of conjugated double bonds which do have electrons in pi-type molecular orbitals in ligands between metal ions should facilitate electron transfer if this theory is correct. It is also easy to extend the idea to overlapping orbitals of resonating systems, such as charge-transfer complexes.

Charge-transfer complexes were first classified by this term by Mulliken (59), who described as examples the colored complexes formed by iodine with aromatic and oxygenated solvents. He explained the absorption

(en) stands for ethylene diamine

of light at 4500-4600 and at 4800 angstroms by postulating the partial transfer of an electron from a solvent molecule to iodine. He also suggested that similar complexes involving other kinds of molecules could be intermediates in chemical reactions. As evidence, he pointed out the reaction of iodine with water to form hypoidous acid and eventually hydroidic acid. In this typical reaction a molecular complex is formed first. Charge separation occurs within the composite, which is then called the "charge transfer complex". The electronic transition involved in the charge separation accounts for the absorption of light. The electronic orbitals of the components of charge transfer complexes must be such that the highest filled orbital of the donor and the lowest unfilled orbital of the acceptor can overlap, thus facilitating the electronic transition.

By postulating the formation of a fairly long-lived intermediate in which an atom such as hydrogen is transferred from reductant to oxidant, it is possible to explain oxidation by two-electron transfer by the bridged complex type of mechanism. The movement of electrons is so very fast with respect to that of the atom transferred, that the argument about whether one or two move at a time in a single molecule is trivial (71).

In electron transfer enzymes which possess metal ions in their active centers it is not possible to tell without

testing whether one or two-electron transfer takes place during redox reactions. If the ion undergoes a change in valence of one equivalent during the course of the reaction it is possible that the enzyme is a one-electron enzyme and that it will release free radicals into the reaction mixture as it acts upon its substrate.

There are, however, enzymes which contain metal atoms in which, as yet, anyway, no valence change has been found during enzymatic action, or as a result of enzyme action. Xanthine oxidase contains molybdenum, but no function has been ascribed to the metal. An attempt (6) to study it by electron spin resonance gave no conclusive results. Many enzymes require Mg^{++} for activity. Phosphorylating enzymes are outstanding examples of these (16, p. 454). Carboxypeptidase contains Zn^{++}, glycylglycine dipeptidase, catalase, and the cytochromes give strong evidence of the capability of one-electron transfer because of the ease with which ferrous ion is oxidized to ferric ion.

The cytochrome system is thought to operate in one-electron steps in the terminal oxidation chain (12, pp. 420, 359). It is known, however, that cytochrome oxidase contains copper (36) as well as heme. The function of the copper is not known at present, but may be an essential part of the oxidase activity (25). It has been difficult to detect valence change in certain copper enzymes during activity. No evidence

has been obtained in the cases of cytochrome oxidase and tyrosinase. In hemocyanin (39), ascorbic oxidase (72), and laccase (60) the copper does function as the electron transfer center of oxidase activity. In laccase (60) and ascorbic acid oxidase (72) activity free radicals are produced from the substrate.

The oxidase of dihydric phenols to quinones is an easy reaction to bring about because the energy barrier that must be overcome to form the "activated intermediate", in this case the free radical, semiquinone, is comparatively low. At high pH some semiquinones are quite stable. The sterically stabilized duroquinone and phenanthraquinone-3-sulfonic acid semiquinones are examples of these (71). Catechol and hydroquinone oxidations occur spontaneously in alkaline solution in the presence of molecular oxygen at ordinary temperatures. The enzymes which increase the velocity of these reactions by a free radical mechanism are following the course of the least resistance, thermodynamically speaking. On the other hand, an enzyme which produces quinone directly from diphenol in one 2-electron step must be capable of a greater energy transfer. The simple presence of a valence-changing metal does not offer an explanation of the difference in these activities. Both kinds of enzymes contain valence-changing metals. A difference in steric configuration, or the manner in which the metal is bound, or the presence of ancillary groups

in the active center might be expected. So far, tyrosinase is unique among catechol-oxidizing enzymes in releasing o-benzoquinone as the first product of its action (53).

The case of oxidation of cresols to diphenols, that is, the hydroxylation reaction, is of an entirely different nature. This type of reaction is an oxidation by the traditional definition of the term inherited from Lavoisier, i.e. combination with oxygen, more than in the modern (Michaelis - 55) sense. The oxygen, in combining with the aromatic nucleus, does certainly gain electrons. It also loses electrons in equal number. Even so, we usually think of the carbon of the aromatic ring as being oxidized, but it is so in only the most formal sense. The electrons of a carbon-hydrogen bond can be thought of as split between a new carbon-oxygen bond and a new oxygen-hydrogen bond. In that sense they are removed further from their carbon and hydrogen nuclei. This movement is partially compensated by the sharing of the new oxygen electrons. The mechanism of this substitution is entirely unclear and it remains to be seen whether attack on the ring hydrogen is made by a hydroxyl or perhydroxyl radical, or by an ion. It is obvious that the enzyme must react by some mechanism which would reduce tremendously the amount of energy required for this reaction, for hydroxylation does not occur with-

out rather energetic conditions in the laboratory.

PARAMAGNETISM AND THE e.p.r. SPECTROMETER

The mechanism of oxidation reactions can be investigated by means of the electron spin resonance spectrometer. This instrument was invented by the Russian, Zavoisky (76) in 1945. It is used to detect paramagnetism in substances and is easier to use and, except for one highly specialized apparatus designed (4, p. 352) to measure triplet state, more sensitive than the Gouey balance (30, p. 38). Paramagnetism is a characteristic property of transition metal ions and organic free radicals, because they contain unpaired electrons. Many elements show paramagnetism under conditions in which their atoms are separated from each other. Conducting metals and certain "odd molecules" show this phenomenon.

In ferromagnetic substances, some or all of the unpaired electrons are permanently oriented in the crystal structure so that their spins are parallel, thus conferring the properties of a permanent magnet, with north and south poles according to the orientation of the electrons, upon the substance. The orientation is brought about by the application of a strong magnetic or electric field to a suitably crystallized substance with enough unpaired electrons to make it susceptible. Some crystal structures are more conducive to magnetic induction than others, special types characteristic of

certain alloys confer these properties upon the substance without the application of an external field, the internal crystalline field being sufficient (Alnico).

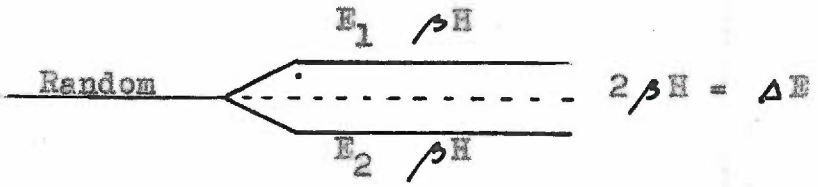
In paramagnetic substances orientation of unpaired electron spins is temporary, brought about by the application of a strong magnetic field and almost immediately dissipated through vibration in the crystal lattice, or as heat in liquids and gases, when the magnetic field is removed. In addition to the spontaneous orientation brought about by the magnetic field, a certain additional amount of orientation can be induced by the application of other forms of energy which have a magnetic component of the right quantum value.

The magnitude of the magnetic field generated by a spinning electron depends upon its angular momentum and its charge (30). Its magnetic moment is also determined by the immediate surrounding field, i.e. its electric and magnetic environment. The energy of spin is quantized in terms of the Bohr magneton:

$$\beta = \frac{eh}{4\pi mc}$$

e is the electron charge, h is Planck's constant, m is the mass of the electron, c is the velocity of light. β equals 0.92731×10^{-20} ergs / gauss of magnetic field applied.

When a magnetic field is applied to free spinning electrons, they occupy only two definite energy levels - one represents orientation of the electron moment with the field of the magnet, and the other against the field of the magnet. Those oriented with the field could be said to be in a lower energy state than those oriented against the field. The separation, or splitting, factor is theoretically exactly 2 for perfectly free electrons. Actually, due to relativity effects, it is 2.0023. For electrons which are not free, the splitting factor may vary greatly from 2. It is designated "g" and the separation in levels is g H.



The number of electrons in these two energy levels is not equal, but the distribution ratio is given by the Maxwell-Boltzmann expression for the distribution of particles into two different energy states:

For N_o free electrons

n_1 are in the higher energy state

n_2 are in the lower state

k is the Boltzmann constant

$$\frac{n_1}{n_2} = e^{\frac{-g\beta H}{kT}} = e^{\frac{-\Delta E}{kT}}$$

Using the first two terms of the McLauren's series approximation

$$\frac{n_1}{n_2} = 1 - \frac{g\beta H}{kT}$$

$$n_1 = N_o - n_2$$

$$\frac{N_o - n_2}{n_2} = 1 - \frac{g\beta H}{kT}$$

$$n_2 = \frac{N_o}{2 - \frac{g\beta H}{kT}}$$

$$n_1 = N_o - \frac{N_o}{2 - \frac{g\beta H}{kT}}$$

$$n_2 - n_1 = \frac{N_o \frac{g\beta H}{2\ kT}}{1 - \frac{g\beta H}{2\ kT}}$$

$$\frac{g\beta H}{kT} \ll 1$$

$$n_2 - n_1 = \frac{N_o\ g\beta H}{2\ kT}$$

Thus we have the extra number of unpaired electrons out of a total of N_o unpaired electrons which will exist in the lower energy state above the number in the higher level. Paired electrons are not considered because their moments cancel each other. Because their orientation is quantized there can be no residual moment.

All of the electrons in the lower energy level can absorb energy and change to the higher level. Those in the higher level can be excited so that they lose energy and drop down to the lower level. The detectable difference in energy absorption comes from the larger number of electrons in the lower energy state. The number in the lower state is increased by a decrease in temperature.

When energy is supplied as electromagnetic radiation, $h\nu = g\beta H$, transitions take place. The magnetic field determines the amount of splitting between the energy levels and, therefore, the quantity of energy necessary for interconversion. Expressed as $h\nu$ this energy determines the frequency of the radiation which will be absorbed at any given magnetic field strength.

The excess number of electrons in the lower energy level is small. It is possible to supply such an intense ray of energy at $h\nu$ that all the electrons go up to the higher level and stay there, so that no more power can be absorbed. The substance is said to be saturated. The electrons in the higher level will dissipate their energy to the crystal lattice, or to the surrounding molecules of solvent, etc. and return to the lower state until the distribution in the two levels is normal for the temperature, but it takes time for this to happen. This time is called the relaxation

time. The relaxation time is very short for transition metal ions in crystals for the dissipation of energy to the lattice is fast, but in the case of free radicals, where the electron occupies a molecular orbital isolated from direct bonds which might absorb the relaxation energy, the time is long and saturation occurs quite easily. It is necessary to keep the temperature low so that there will be a tendency for more electrons to remain in the lower energy levels.

Theoretically, the absorption frequency can be set at any convenient value by adjusting the magnetic field to a level which will bring absorption in any given electromagnetic region. Practically, magnets must be chosen within the limits of homogeneity of field, size, etc., which are available. Fields from 3,000 to 13,000 gauss are used. The radiation then falls between 9,000 to 36,000 Mc/sec. The most popular is the 9,000 Mc/sec. frequency radiation which has a wave length of 3 cm., and requires the 3,000 gauss magnet. The range extends from 1 mm. to 30 cm. and is the microwave region.

Thus, by placing a sample of reaction mixture in a magnetic field and allowing microwave radiation to play upon it of the right frequency and of not too great an intensity, we have only to detect a decrease in the radiation reflected from or transmitted through the sample to determine that the sample contains free radicals. The power absorbed by the sample is a measure of the number of free spins present in the

sample.

That is, if the excess electrons present in the lower energy level are the only ones whose transitions can be detected, the number of transitions taking place in the unit time would be

$$p \frac{N_o g \beta H}{2 kT}$$

If the magnetic moment of each electron is ½ g β, the total magnetic moment is then

$$p \frac{N_o g^2 \beta^2 H}{4 kT}$$

where p is the probability of the transition

If ω is the sweep frequency of the magnetic field, the above expression becomes

$$p \cdot \frac{N_o \hbar \omega}{2 kT} \sim P$$

At ω which is near ω_o

$$P = p \frac{N_o \hbar^2 \omega_o \omega}{2 kT}$$

represents the power absorbed. ω_o is exactly the resonance absorption.

For any transition $M_s \longrightarrow M_z - 1$, the probability that it will take place is

$$P_{M_z} = \frac{\pi}{4} \gamma^2 H_1^2 (S + M_z)(S - M_z + 1) g (\omega - \omega_o)$$

from standard radiation theory. Then in going from $M_z \rightarrow M_z - 1$ the power absorbed is

$$P_{M_z} = P_{M_z} \frac{N_o \hbar^2 \omega_o \omega}{2\,kT}$$

and combining,

$$P_{M_z} = \frac{\pi \gamma^2 H_1^2 N_o \hbar^2 \omega\omega_o (S + M_z)(S - M_z + 1)\, g(\omega - \omega_o)}{8\,kT}$$

Then, for all possible transitions, the power absorbed is

$$P_A = \frac{\pi N_o \gamma^2 H_1^2 \hbar^2 \omega\omega_o}{8\,kT}\, g(\omega - \omega_o) \sum_{M_z = S}^{M_z = S} (S^2 - M_z^2 + S + M_z)$$

$$\gamma = \frac{g\beta}{\hbar} \qquad S = \tfrac{1}{2}$$

$$\sum_{M_z = \frac{1}{2}}^{M_z = \frac{1}{2}} = 1 \qquad P_A = \frac{\pi N_o H_1^2 g^2 \beta^2 \omega\omega_o}{8\,kT}\, g(\omega - \omega_o)$$

$g(\omega - \omega_o)$ is the entire absorption function which integrates to unity, so that the total number of unpaired spins in the sample is

$$N_o = \frac{8\,kT}{g^2\beta^2} \cdot \frac{P_A}{\pi H_1^2 \omega\omega_o} = 1.02 \times 10^{31} \frac{P_A T}{H_1^2 \omega\omega_o} =$$

$$\text{integrated intensity} \cdot \frac{T}{H_1^2 \omega_o^2} \cdot \text{constant}$$

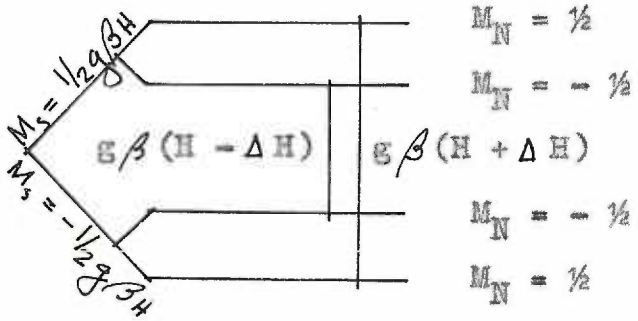
The constant includes the gain of the amplifier system and the units in which intensity is measured and is evaluated by measuring a standard sample. The integrated intensity is equal to the power absorbed

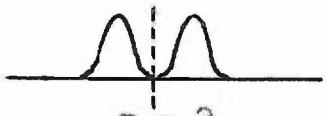
as the magnetic field sweeps through resonance at the wave length fixed by the microwave source of the apparatus.

The absorption of energy by the unpaired electrons of free radicals is not a simple function of the carefully measured magnetic field applied. The electron is influenced by the other dipoles in the molecule or crystal of which it is a part. In liquids or amorphous solids where the molecules are free to turn and not limited to short vibrational motions, the magnetic components associated with orientation of individual molecules in a lattice are smeared out and only the effect of individual dipoles within the atom or molecule remain. These dipoles arise from spinning atomic nuclei. As the electron passes near them in its orbit its immediate magnetic environment is changed a little and it absorbs energy at a slightly different wave length, or at constant wave length but different value of applied magnetic field. There is an apparent change in g-value or splitting factor. The time the electron spends in the neighborhood of the nucleus determines the intensity of the lines that result from orientation with or against the field of the nucleus itself. These lines are always less intense than the unsplit line. Not all atomic nuclei produce this effect. Only those which have a magnetic moment associated with their spin bring about this hyperfine

structure in the electron spin resonance lines. Hydrogen and nitrogen are the most common magnetic nuclei found in the free radicals of biological systems. Carbon and oxygen, that is, the ordinary isotopes of carbon and oxygen, do not show any splitting.

For one dipole, say a proton, the energy level splitting can be represented

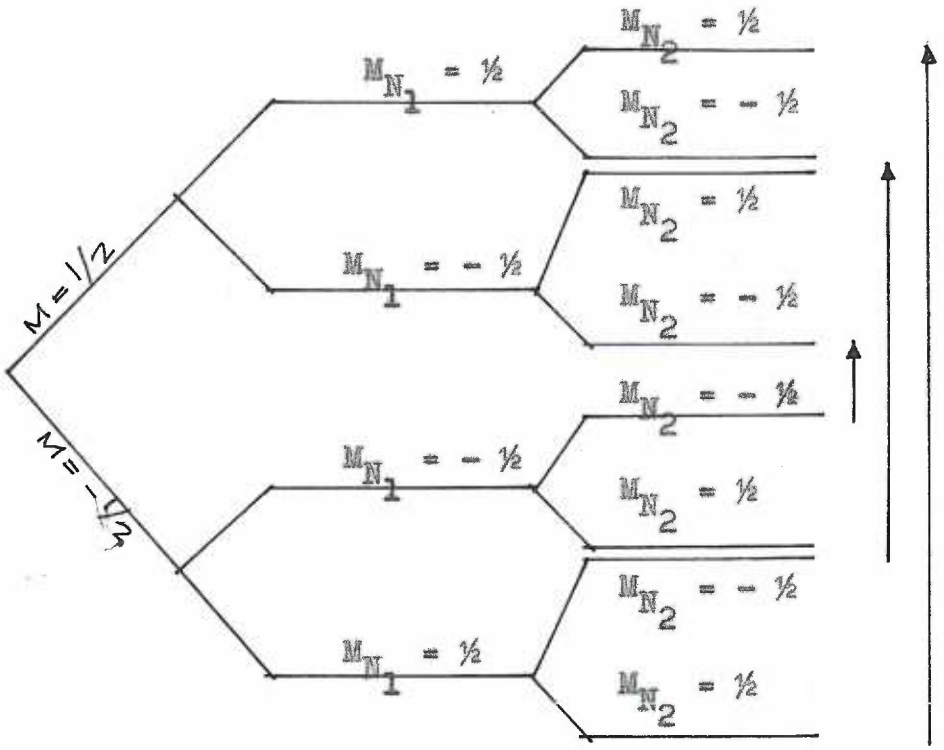


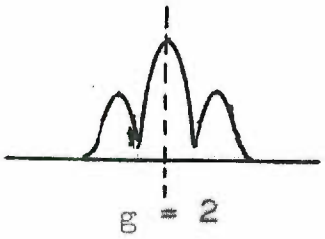


Signal

Each of the two electronic energy levels is split as shown into two sublevels, depending upon the orientation of the magnetic moment of the proton. By the selection rules, only transitions in which $M_N = 0$ are allowed. The line $g\beta H$ disappears and two new lines half as strong appear, $g\beta(H + \Delta H)$ and $g\beta(H - \Delta H)$.

For two protons in a molecule the effect is compounded and the diagram shows the energy state.





Signal

There are then three lines of intensity 1:2:1. For more protons still more splitting results, with intensities of the lines following the binomial law, that is, for three protons, 1:3:3:1. If, however, nuclei of different magnetic moment interact, the number of lines is given by the formula (2n S + 1), where n is the number of nuclei of spin S. One can tell something about the structure of a free radical from the hyperfine splitting of the electron spin

resonance spectrum.

From the foregoing it is seen that detection, identification and quantitative estimation of free radicals by the electron spin resonance spectrometer in reaction systems could help to elucidate and formulate the reaction mechanisms of enzymes which produce free radicals either directly or as a byproduct in their reactions with their substrates. It is also possible to determine the valence state of any transition metal in the enzyme, and to detect free radicals in the body of the enzyme itself.

There are, of course, limitations to the usefulness of the electron spin resonance spectrometer in the applications mentioned above. If the free electrons of a biradical are too close together they will interact to broaden and smear out the signal. They may then go undetected. Molecules in the triplet state often fall into this catagory. There is also a lower limit to the concentration of free spins which can be detected. The theoretical limit is 10^{11} spins per gram of substance which is about 10^{-7}M free radical in .01M solute* in aqueous solutions. The presence of water does interfere with the method because the water absorbs the electrical energy of the microwave radiation, lowering the Q value or capacity of the cavity. Townsend's flat cell (8) helps to reduce the effect of the water by making its thickness in the direction of the

*M stands for molar throughout this paper.

electric field as small as possible. Quartz is generally used for sample containers because glass is likely to have paramagnetic impurities.

APPLICATION OF e.p.r. SPECTROMETRY TO THE STUDY OF BIOLOGICAL SYSTEMS

The first application of electron spin resonance methods to the detection of free radicals in biological systems was made by Commoner, Townsend and Pake (14) of Washington University, St. Louis in 1954. They lyophilized a number of plant and animal materials and found them to contain an appreciable content of free radicals. This work touched off widespread investigation of the free radical content of all sorts of biological materials by the electron spin resonance technique.

Commoner's group continued investigating first, whole cellular materials, and then mitochondrial particals and enzyme systems. In 1956 (10) they showed the induction of paramagnetism in chloroplasts by light. They concluded that this property could be associated with the triplet state supposed to be characteristic of activated chlorophyll. The following year (9) they published results showing that living Chlorella cells produce free radicals during active electron-transport. They then showed that the alcohol dehydrogenase-DPN-alcohol system gives a free radical intermediate which

can also be generated from acetaldehyde-DPNH-enzyme side. The lactic acid dehydrogenase and glucose-6-phosphate systems gave similar results. Comparable experiments with cytochrome reductase, cytochrome c and cytochrome oxidase with reduced DPN and oxygen gave comparable results. In 1958 (13) these studies were extended to lactic oxidase decarboxylase, glyceraldehyde phosphate dehydrogenase, and aldolase. They found that aldolase, which is not associated with electron transfer, does not give an e.p.r.signal when mixed with its substrate. All the flavoprotein enzymes studied gave a transient free radical intermediate when acting upon their substrates which is similar in g-value and half-width to the radical obtained when flavoprotein enzymes are illuminated in the absence of substrate, and it was concluded that the free radical comes from the prosthetic group. The steady-state concentration of this radical during the reaction was varied by regulating the rate of enzyme activity.

Commoner's group then (12) proceeded to investigate the FMN and flavoprotein enzyme free radicals produced by light. They found that, while the free radicals were quite stable in the dark after they had been formed they were completely destroyed by oxygen and not regenerated by light until the oxygen was removed. The g-value and hyperfine structure were the same as those of free radicals produced by mixing FMN and DPNH or borohydride. Lippincott, et al. (42) showed that

flavins partially reduced by substrate give this same free radical, and that it disappears in the presence of an electron acceptor such as oxygen.

Commoner and Hollocher (26)(11) published in 1960 the results on their work with heart muscle mitochondrial particles, showing that the free radicals which they observed were attributable to the activity of succinic dehydrogenase, and were probably identified as the complex between the flavoprotein and substrate.

Sogo, Pon and Calvin (67) in 1957 studied the e.p.r. spectrum of spinach chloroplasts. They concluded that because the decay of the radicals is retarded by cooling it is not the triplet that gives the signal, but trapped electrons or dissociated bonds brought about by the action of light. The narrow band indicated a pi-type orbital for the unpaired electron, removed from lattice interaction.

Meanwhile, Bray, Mälmstrom and Vänngård (6) in 1959 published the results of their work on xanthine oxidase, a flavoprotein enzyme, from a different point of view. They studied the changes in the valence of iron and molybdenum atoms during enzyme activity. They did get the expected semiquinone from the flavin-adenine-dinucleotide but did not study it further. They recorded a resonance which they attributed to a special kind of ferrous iron, or to molybdenum +3 or +5. They thought Mo +5 most probable. They considered this

evidence for the participation of free radicals in enzyme reactions.

Mälmstrom, Mosbach and Vänngård (46) studied the copper of fungal laccase and found definite evidence of a change in valence from Cu II to Cu I during reaction. The pure enzyme showed the typical signal of Cu II which was greatly reduced upon the addition of catechol. When the substrate was exhausted the copper signal returned, also the blue color which faded during enzyme activity.

The metal ions found in mitochondrial particles, succinic and DPNH dehydrogenase were studied by Beinert and Sands (2). They found some non-heme ferric iron which gave a signal at g = 4.3 which also shows possible reduction by substrate to an iron giving a signal at g = 2. The signal was not positively identified and may not be iron. In any case the signal at g = 4.3 decreased. The new signal was well differentiated from the flavin free radical signal. In another publication (65) it was stated that cytrochrome oxidase gives a copper signal, and that all the signals mentioned in the previous paper are found in beef heart mitochrondia and in particles derived from them.

The work of Gordy (22, 23) was concerned with the effect of ionizing radiation upon proteins. He and his group have studied the nature of free radicals produced in proteins and the protective action of sulfur-containing amino acids and other substances. They proposed

the idea that proteins can conduct unpaired electrons or "holes" along the polypeptide chain for many angstroms to "traps" where they are stabilized.

The detection of substrate free radicals in enzyme systems by Yamazaki, Mason and Piette (72) gave the first evidence that Michaelis (55) and George (22) were right in speculating that when enzymes act to produce a one-electron transfer, short-lived free radicals are set free in the reaction medium. The technique used in this research was also new, for instead of freezing samples at various times during the reaction, these investigators employed a flow apparatus designed by Berger which could be used to give a continuous sampling of the reaction mixture at a given time, or to introduce the sample into the cell early in the course of the reaction, and by stopping the flow, to observe the whole course of the reaction. Even though the free radicals were very short-lived, they were found to accumulate to a measurable steady-state concentration, and peroxidase and ascorbic acid oxidase were proved to act by a free radical mechanism. Employing the same technique with Chance's manually operated flow apparatus, Nakamura (60) found that laccase also acts by a free radical mechanism.

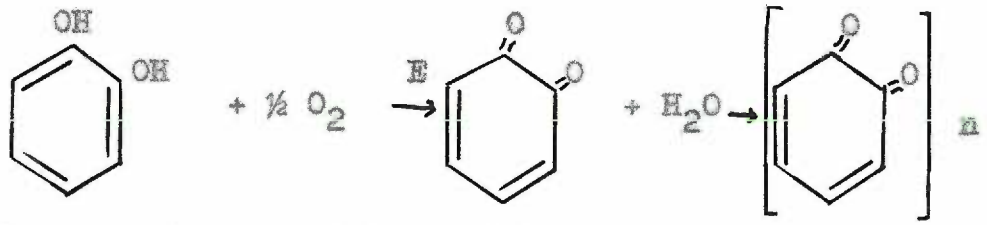
THE ENZYME TYROSINASE

Tyrosinase is very widely distributed in nature in both the plant and animal kingdoms. It is the enzyme (50, 51) which catalyzes the reaction between molecular oxygen and certain phenolic substances in tissues to form colored oxidation products of high molecular weight known as melanins. These melanins, when united with the protein of the tissue, are the pigments which determine the colors of hair, eyes, fur and feathers. Their production accounts for the browning of fruits and vegetables when injury exposes the inner tissues to the air, and for the color of the protective coatings of insects. Squid and octopus ink are melanoproteins. Melanogenesis causes the tanning of human skin when it is exposed to sunlight.

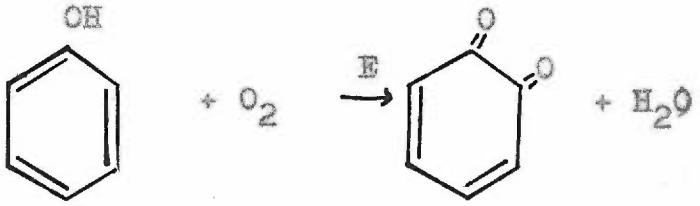
After careful isolation and purification from the tissues in which it occurs in large amount, such as potatoes or mushrooms, tyrosinase is obtained as a pale yellow protein (75) of molecular weight 100,000 containing 0.2% copper. Calculation shows this to be four copper atoms per molecule. It is electrophoretically homogeneous. Its sedimentation constant in the ultracentrifuge Frieden (21) reports as 7.3×10^{-13} with $D_{20} = 7.7 \times 10^{-7}/cm^2\ sec^{-1}$. He later (31) reports 8 Svedberg units for highly purified tyrosinase in .005 M phosphate buffer, pH 8. Frieden (31) gives the activity of his preparation as 10^4 of his units per ml. per unit of optical density at 280 m. The best prepara-

tions of Yasunobu, Thomson and Mason (75) contain 3200 Miller-Dawson units per mg. dry weight.

The enzyme has two characteristic activities (45) which have never been completely separated. These are the catecholase and cresolase activities. The latter is reduced upon purification of the enzyme. The catecholase activity oxidizes diphenols to quinones (47, 48, 49, 50, 51), which then polymerize to form melanins, according to the following overall equation:



The cresolase activity catalyzes oxidation of monophenols to diphenols and then immediately brings about their further oxidation to quinones:



The cresolase activity of the enzyme never occurs without catecholase activity. When no diphenolic substrate is present, the monophenolic activity shows a characteristic time lag period which can be eliminated by introduction of diphenol at the beginning of the reaction, by addition of reducing agents such as ascorbic acid, or by removal of a naturally occurring inhibitor by means of DEAE-cellulose column (31). Thus

it is generally believed that these two activities are attributes of the same enzyme protein and not evidence that tyrosinase is a mixture of two different enzymes. The catecholase/cresolase ratio varies with different preparations and with the degree of purity. Mallette et al (45) report a ratio of 0.5 in the crude mushroom extract while Yasunobu et al (75) observed 230-250 for their purified preperation.

Frieden (31) reports that the Km for his purified enzyme for catechol is 1×10^{-5} M, whereas for the crude preparation it is 5×10^{-5}M. With tyrosinase as a substrate the Km for the purified enzyme is 4.8×10^{-5}M and for the crude 7.7×10^{-5}M. Ingraham (29) reports that the Km for oxygen of his prune tyrosinase is 1.5% of one atmosphere at low catechol concentration.

Many different methods have been used for the isolation and purification of tyrosinase. It is usually prepared from either mushrooms (Psalliotta campestris) or potatoes. Mushrooms are a better source because of the occurrence of globulin-like materials in potatoes which make separation difficult (62). Mammalian tyrosinase has been prepared by Brown and Ward (7).

The first reliable production procedure from mushrooms was devised by Keilin and Mann (37). The enzyme was extracted from the mushrooms with water by grinding with sand. It was purified by ammonium sulfate fractionation, absorption on calcium phosphate gel, elution with disodium phosphate, and precipitation

of inactive colored proteins by lead acetate. After sufficient repetition with alternation of the above procedures, a colorless, very active preparation was obtained in 2.8% yield based on the activity of the original water extract. The method was tedious and exacting and improvement of the yield was obviously desirable.

Kubowitz (39, 40) prepared tyrosinase from potato peelings obtaining a pure, pale yellow enzyme. He found that dialyzing it against cyanide destroyed its activity, and that it could not be reactivated by dialyzing the cyanide out against water. He proved that the activity could be restored by the addition of copper salts, and that the activity of the enzyme is proportional to its copper content. He stated that copper is the prosthetic group of the enzyme, that its valence change is responsible for the enzyme activity. He showed that it combines with and is inactivated by carbon monoxide. He indicated that it combines with the CO only as it acts upon catechol, and that only half of the copper takes up CO, or two atoms of copper bind one CO molecule.

Mallette, Lewis, Ames, Nelson and Dawson (45) made an acetone powder of the mushrooms by grinding them into acetone cooled with dry ice. After collecting the pulp they extracted it with water. By careful ammonium sulfate fractionation they obtained two

preparations which differed from each other in the catecholase/cresolase activity ratio. They used lead and barium acetates to remove colored impurities and absorption of alumina with elution in buffer to further purify the enzyme. They eventually obtained electrophoretically homogeneous proteins, one high in cresolase activity and the other higher in catecholase activity. Both preparations had both activities, however.

Kertesz and Zito (34) made their acetone powder by homogenizing mushrooms in acetone at -20°C to get a light-colored material which they extracted quickly with 30% actone. Addition of pure actone at -20° brought down a protein precipitate which was collected and dissolved in a minimum volume of water. If all these first steps were carried out in one day the yield was maximum. Calcium acetate was added to the aqueous solution to precipitate extraneous proteins. Heating to 46° helped to accomplish the same purpose. Ammonium sulfate and acetone fractionations led to an electrophoretically homogeneous, pale yellow enzyme in 10% yield.

Frieden and Ottesen (21) modified the method of Kertesz and Zito by taking the crude enzyme after treatment with calcium acetate, dialyzing it against various salts, and placing it upon a column of DEAE-cellulose according to the method of Brown and Ward. They eluted the enzyme with 0.08M phosphate buffer, pH 8, reporting a yield of 28% based on the activity

of the acetone precipitate. They reported very high purity. Frieden and Karkhanis (31) have studied a protein fraction eluted from the column before the enzyme with 0.04M phosphate buffer, pH 8, and claimed that it is the tyrosinase apo-enzyme. They stated that this inactive protein can be incubated with dilute $CuSO_4$ for five hours to reconstitute its tyrosinase activity. They also pointed out the similarity between this protein and the apo-enzyme of Kubowitz (32).

The study of this enzyme is complicated by the proliferation of different methods of estimating catecholase activity. Manometric methods which were used at first, and were found satisfactory for the cresolase activity, gave such variable and confusing results that development of a more reliable technique was mandatory. The first attempt made to standardize estimation of catecholase activity was in 1938 by Keilin and Mann (37) who defined the purpurogallin number as the amount of enzyme which will produce 100 mg. of purpurogallin from pyrogallol in 5 minutes at 20°C. The purpurogallin was estimated colorimetrically in an ether extract of the reaction mixture. This technique soon proved to be too inaccurate because of reaction inactivation of the enzyme over the period of measurement to be satisfactory.

Miller and Dawson (56, 57, 58) then developed the "chronometric method", which has been standard until recently. The chronometric method eliminates the

problems of reaction inactivation, variable oxygen consumption, and accumulation of reaction products in the solution. A known amount of ascorbic acid is added to the reaction mixture in the beginning. It reacts with the o-benzoquinone as it is produced and converts it back to catechol, itself being oxidized to dehydroascorbic acid. Tyrosinase does not catalyze the oxidation of ascorbic acid by oxygen (49). When the ascorbic acid is all used, quinone appears in the solution. Its presence is detected by a continuous sampling technique in which it releases I_2 from KI in an external indicator solution containing starch. The rate of disappearance of ascorbic acid is equal to the rate of quinone production. The unit is defined as that amount of enzyme which will produce 1.49 moles of o-benzoquinone from catechol per second at optimum catechol concentration, air saturation, and pH 5.1.

El Bayoumi and Frieden (18) published a spectrophotometric modification of the above method in which they followed the disappearance of ascorbic acid in the reaction mixture by noting the decrease in absorption at 265 mμ. They defined the unit as that amount of enzyme which would produce a change of .001 unit of optical density per minute. Since the concentrations of reagents were entirely different, the pH 7 instead of 5.1 comparison of activity units with the Miller-Dawson unit is quite impossible.

	Miller-Dawson Concentrations	El Bayoumi & Frieden Concentrations
ascorbic acid	10^{-5}M	2×10^{-4}M
catechol	5×10^{-3}M	1×10^{-3}M
ethylenediamine tetraacetate	0	present

The old unit has been calculated by J. O. Alben (1) to be approximately 2.3 times the new one. Frieden and Karkhanis (31)(32) have recently published a still newer unit based on the use of tyrosine instead of catechol. They seemingly make no distinction between cresolase and catecholase activities of the enzyme.

The first attempt to explain the mechanism of tyrosinase action was made by Onslow and Robinson, who postulated the production of hydrogen peroxide by the enzyme, which was then the actual oxidizing agent. Since no hydrogen peroxide has ever been found in tyrosinase reaction mixtures and catalase has no effect upon the activity of the enzyme, this explanation has been discarded (62).

The overall stoichiometry of the reaction was worked out by Dawson (62), Mason (50) proposed several possible mechanisms in his review in 1957. In accordance with the theories of Michaelis, he proposed the formation of a ternary complex of oxygen, enzyme and catechol in two bimolecular steps, which subsequently decomposed to give quinone, water and oxygenated enzyme.

The oxygenated enzyme could then react with monophenols to give diphenols, enzyme and water.

This explanation also leaves room for a mechanism of reaction inactivation involving random cleavage of the complex at a different bond so as to denature the protein. It is thought that copper is involved in the binding of substrate and oxygen because the enzyme is inactivated when the copper is removed or sterically covered by chelating agents.

$$E + O_2 \rightleftharpoons EO_2$$

$$EO_2 + AH_2 \rightleftharpoons EO_2 \cdot AH_2 \rightleftharpoons EO + H_2O + A$$

$$EO + BH \rightleftharpoons E + BOH$$

The enzyme has an affinity for oxygen. It is possible, however, for it to combine with the substrate first, and then the oxygen. Any mechanism must account for the simultaneous catecholase and hydroxylation activities.

There are other oxidative enzymes which act upon their substrates by removing one electron at a time leaving free radicals to accumulate in the reaction mixture. These substrate free radicals then decompose or react with other substances according to their nature and the composition of the reaction mixture. The possibility of this kind of enzyme action is discussed by Michaelis and was first proposed for peroxidase by George (22). It remained for Yamazaki, Mason and Piette (72, 73) to obtain the first evidence that free radicals were indeed produced in solution by peroxidase during peroxida ic oxidation of hydroquinone and

ascorbic acid. The p-benzosemiquinone free radical was identified by its hyperfine structure (68) in the electron spin resonance spectrometer and its appearance and accumulation in the reaction mixture was followed at room temperature by use of a flow technique which enabled them to observe changes in the reaction mixture very soon after mixing. The ascorbic acid free radical was likewise identified and the free radical mechanism for peroxidase was experimentally confirmed. The same kind of experimental work on ascorbic acid oxidase, a copper protein, showed its action to be of the same type (74). Nakamura (61) demonstrated similarly the fact that laccase belongs to the same group.

We have investigated the possibility that tyrosinase might also be one of the oxidative enzymes that acts by removing electrons one at a time from substrate molecules. It is a copper protein, but unlike all other copper enzymes does not show the blue color characteristic of cupric ion at any time, even during reaction. There has been much debate about the valence of copper in tyrosinase. Kertesz (33) has stated that the copper of the enzyme is always in the cuprous form, but Krueger (38) insists that there is both cuprous and cupric ion in this enzyme. They both used the same method of estimating cuprous ion, that of chelation by biquinoline at pH 1.9. If the enzyme acts by a free radical mechanism, it is hard to see how it would be possible without a change in valence of the copper.

The research which is the subject of this thesis was undertaken with the object of determining whether or not tyrosinase acts by a free radical mechanism. We also wanted to find out something about the kinetics of the reaction, the nature of the copper binding in the enzyme and how the substrate and oxygen are held in the complex if such a complex is formed. We have used the electron spin resonance spectrometer in the attempt to detect free radicals in substrate and enzyme and cupric ion in the enzyme.

MATERIALS AND EQUIPMENT

THE ELECTRON SPIN RESONANCE SPECTROMETER

The measurement and characterization of free radicals in the work to be described were obtained by the use of a Varian V-4500 X-band spectrometer. It was equipped with a Varian F-8 Fluxmeter and a Hewlett-Packard frequency meter. A flat quartz cell first designed by Townsend (9) was placed in the cavity so that its widest dimension was perpendicular to the faces of the pole pieces of the magnet. It proved to be about 0.4 mm. thick, inside measurement, but is probably not uniform throughout its length. The flat part is placed in the center of the cavity so that the magnetic lines of force of the microwaves meet and pass through the sample in a thin narrow in the center of the cavity. This cell is used for aqueous solutions at room temperature so that flow systems can be used without introducing so much water that the Q of the cavity is seriously reduced.

THE GAS-DRIVEN FLOW SYSTEM

The quartz cell was fitted with a lucite 4-jet mixing chamber* by means of the shortest possible connection (Fig. III). The volume of solution between the center of the cell and the mixing jets was 0.2 ml. The two inlets to the mixing chamber were connected through

*Kindly loaned to us by Varian Associates

solenoidsvalves to bottles containing the solutions used in the reactions studied. The flow system was similar to that designed by Berger. The bottles were fitted with rubber stoppers which could be wired down solidly when gas pressure was applied. Gas was introduced into the tops of the bottles from a nitrogen tank equipped with a reservoir bottle and manometer. Pressure was built up in the system according to the flow rate desired. The solenoids were controlled by a single switch so that flow could be started or stopped immediately. The top of the quartz cell was connected to rubber tubing to carry off the spent reaction mixture. This waste line carried a thermocouple. The flow rate was measured by timing the collection of a known volume of fluid from the waste line.

Figure I The microwave bridge and frequency meter of the electron spin resonance spectrometer. The klystron tube is enclosed in the cabinet and the microwaves are conducted by means of a waveguide to the cavity where the sample is located. The frequency meter contains another cavity with a crystal detector. When its dimensions are changed by means of the black ring on top, perfect tuning is indicated by a dip in power transmission and the frequency is read directly from the graduations on the cavity adjustment. The meter has been calibrated against the standard radio frequency broadcast by the Bureau of Standards.

Figure I

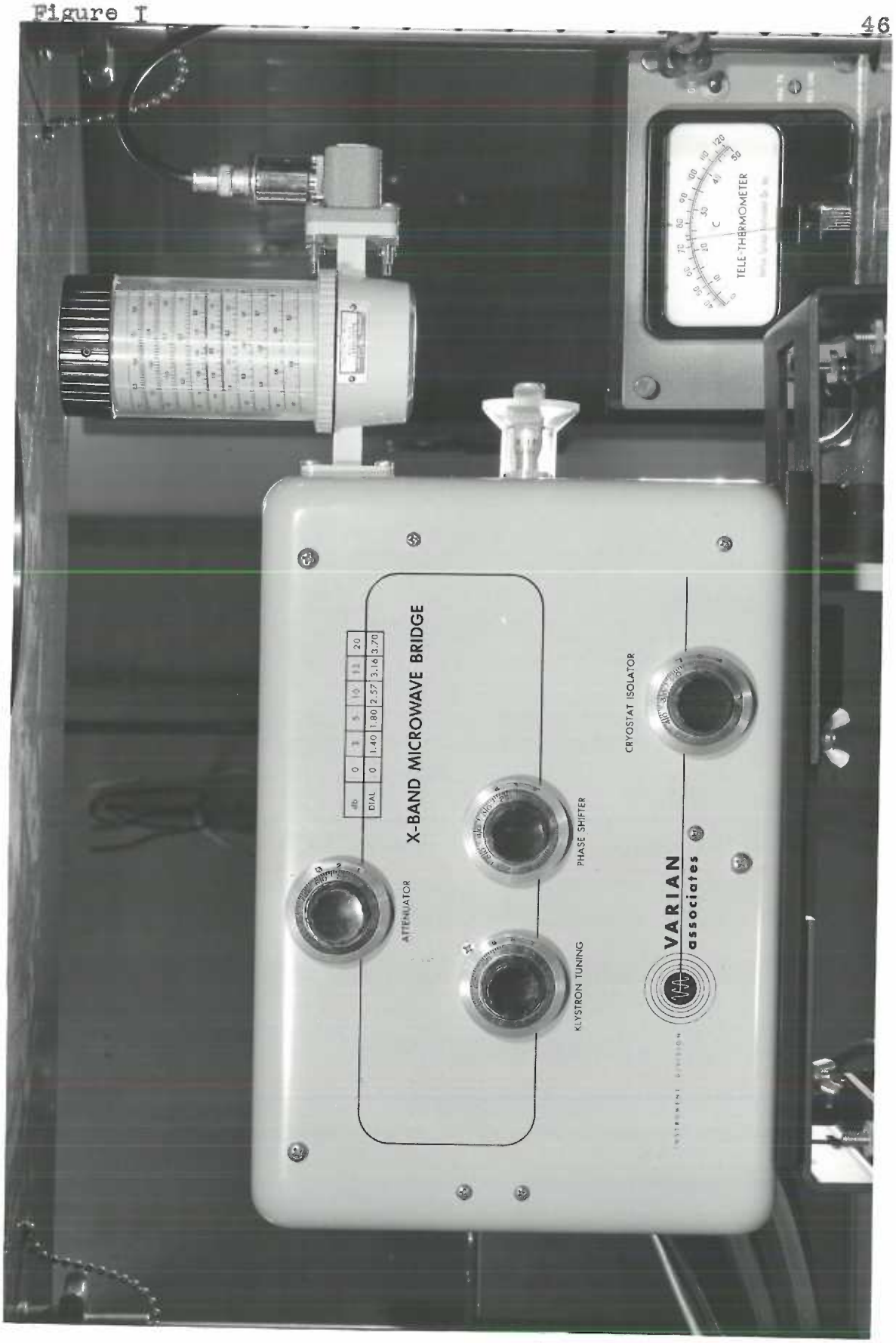

X-BAND MICROWAVE BRIDGE
ATTENUATOR
db 0 3 5 10 15 20
DIAL 0 1.40 1.80 2.57 3.16 3.70
PHASE SHIFTER
KLYSTRON TUNING
CRYOSTAT ISOLATOR
VARIAN associates
TELE-THERMOMETER
C

Figure II The gas-driven flow system. In use, the bottle on the left is filled with catechol solution saturated with nitrogen. The bottle on the right contains the enzyme solution saturated with oxygen. In the center is the solenoid switch which controls the two valves leading to the mixing chamber. A manometer aids in regulating the nitrogen pressure on the reservoirs.

Figure II

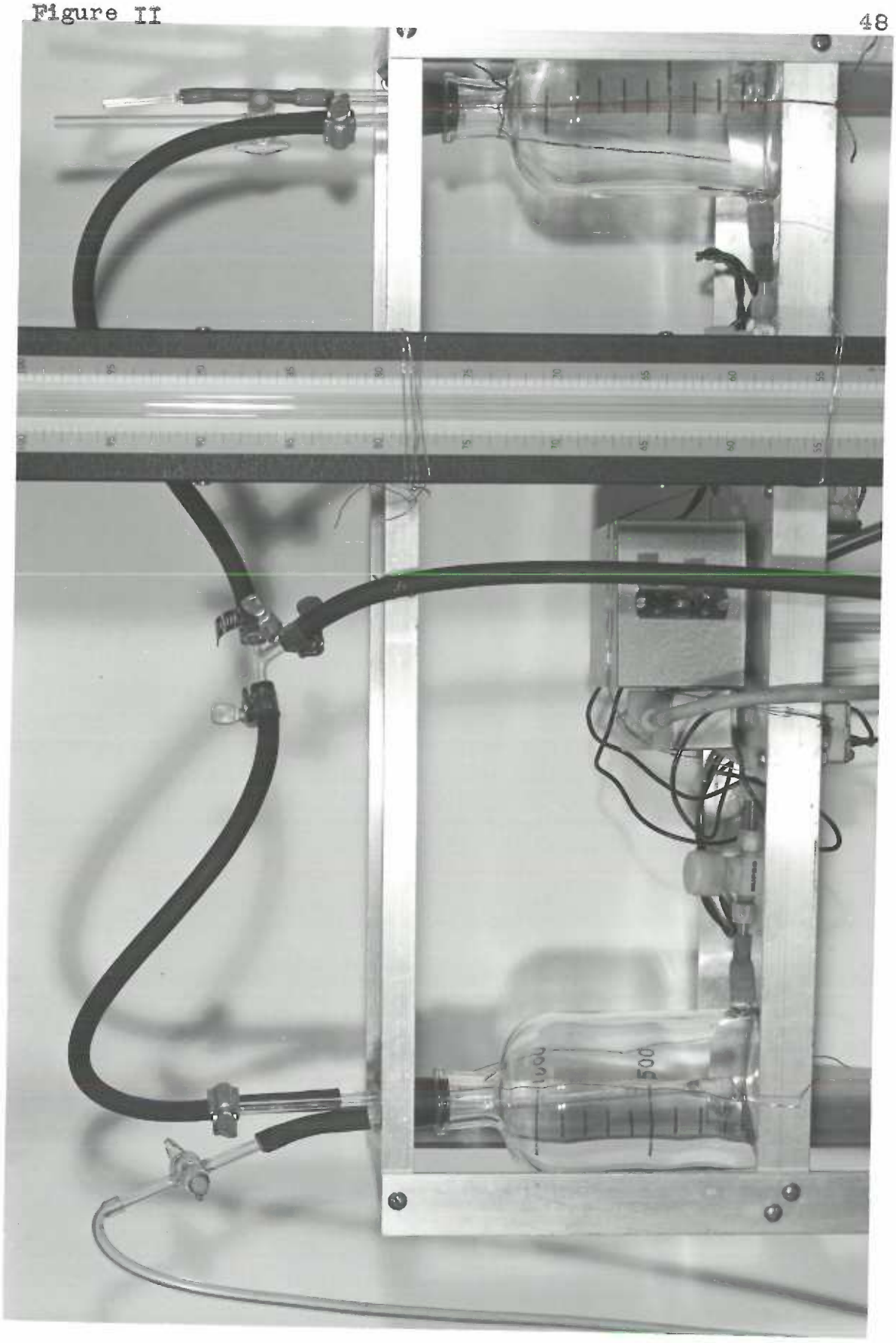


Figure III The flat quartz cell, similar to that of Townsend, with mixing chamber for the flow apparatus attached. The four-jet mixing chamber is in the circular part of the lucite piece just above the quartz portion of the assembly.

Figure III

Figure IV The recirculation apparatus. Here the brass cavity of the spectrometer is shown between the pole-pieces of the water-cooled magnet. The wave-guide leading down from the microwave bridge is seen at the top of the picture. The quartz cell is in position in the cavity and the connection on top leads to the Sigma pump in the foreground. The tube from the bottom of the cell leads into a beaker to which the recirculated reaction mixture also returns from the pump. A fritted glass filter stick delivers gas in small bubbles to the solution from the tank in lower right.

Figure IV

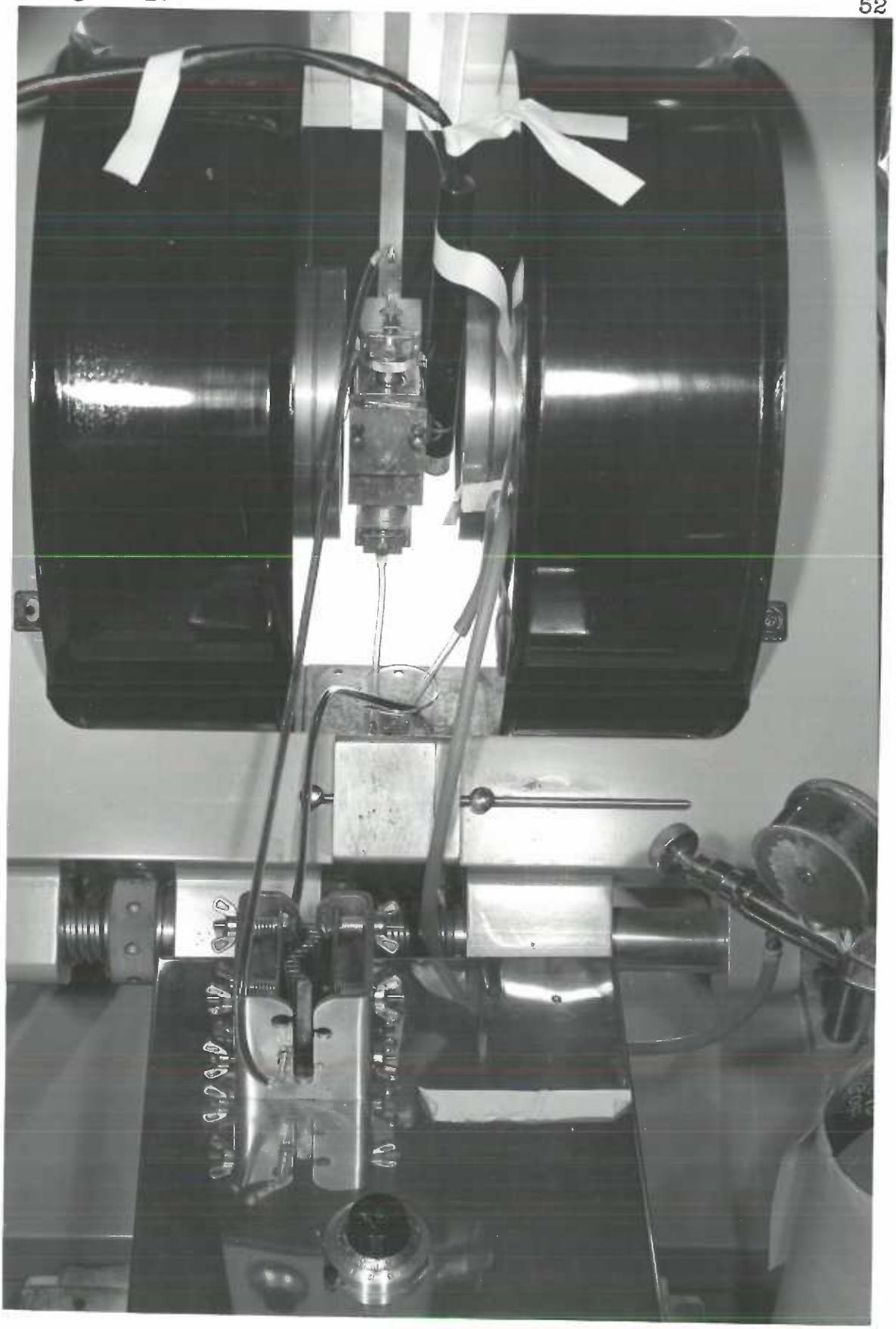


Figure V A close-up view of the cavity and gas-exchange reservoir. Between the cavity and the right pole-piece of the magnet is the probe of the fluxmeter. The fluxmeter measures the magnetic field. The probe contains a standard sample of .25 M gadolinium chloride in water which gives a characteristic nuclear magnetic resonance at known values of frequency and magnetic field. When resonance shows on an oscilloscope which is connected to the probe, but not in the picture, and the frequency is measured by the frequency meter, the magnetic field can be calculated. A frequency meter on the probe would make this apparatus accurate to four decimal places in determination of the g-value of a signal.

Figure V

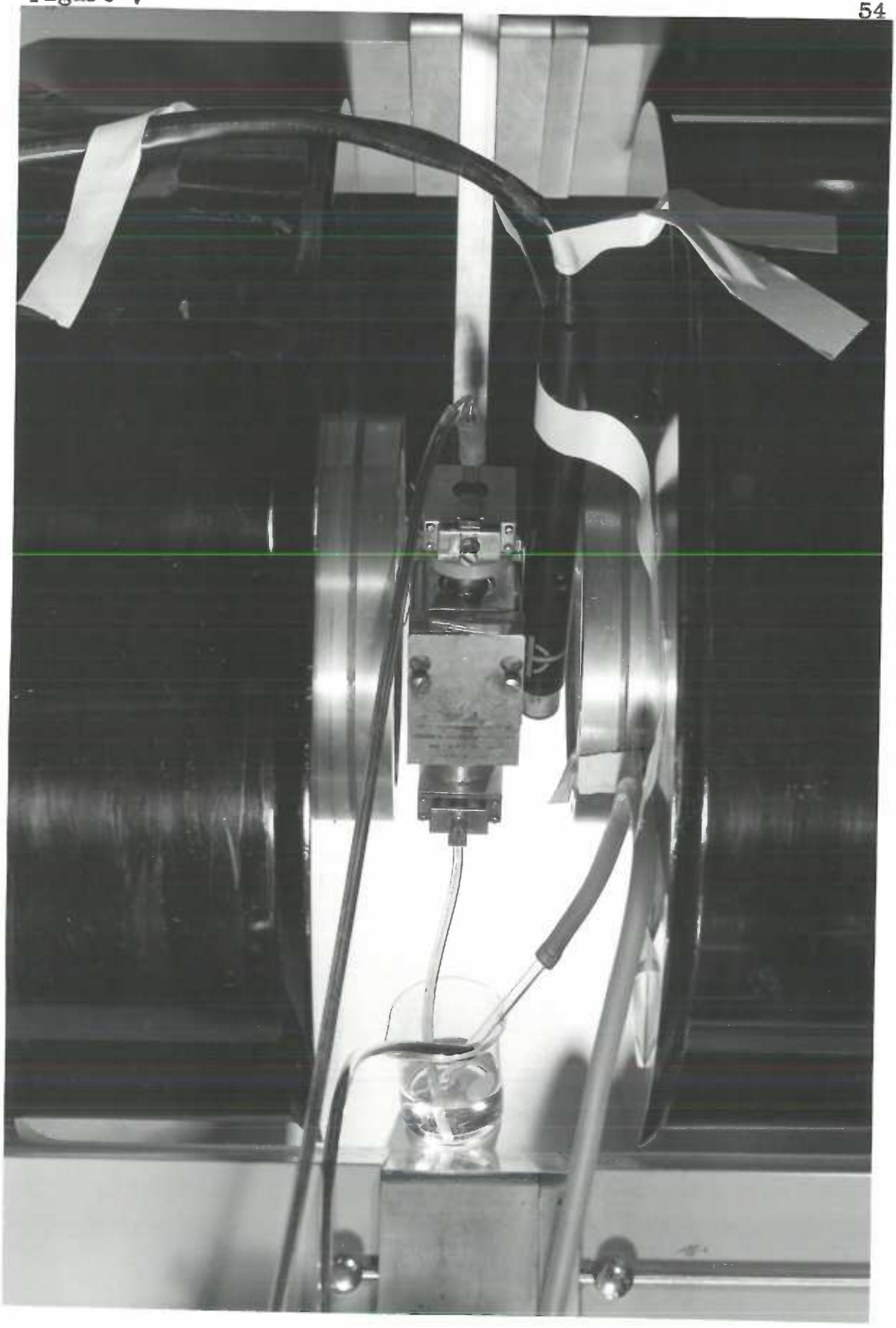


Figure VI The cavity and gas-exchange reservoir from the reverse side. This view shows the wave guide as it enters the cavity and the electric cord leading from the sweep coils to the electronic control system. The standard-containing tip of the magnetic probe is clearly visible. The capillary intake from the reservoir to the cell and the efficient gas-bubbler are shown in position.

Figure VI

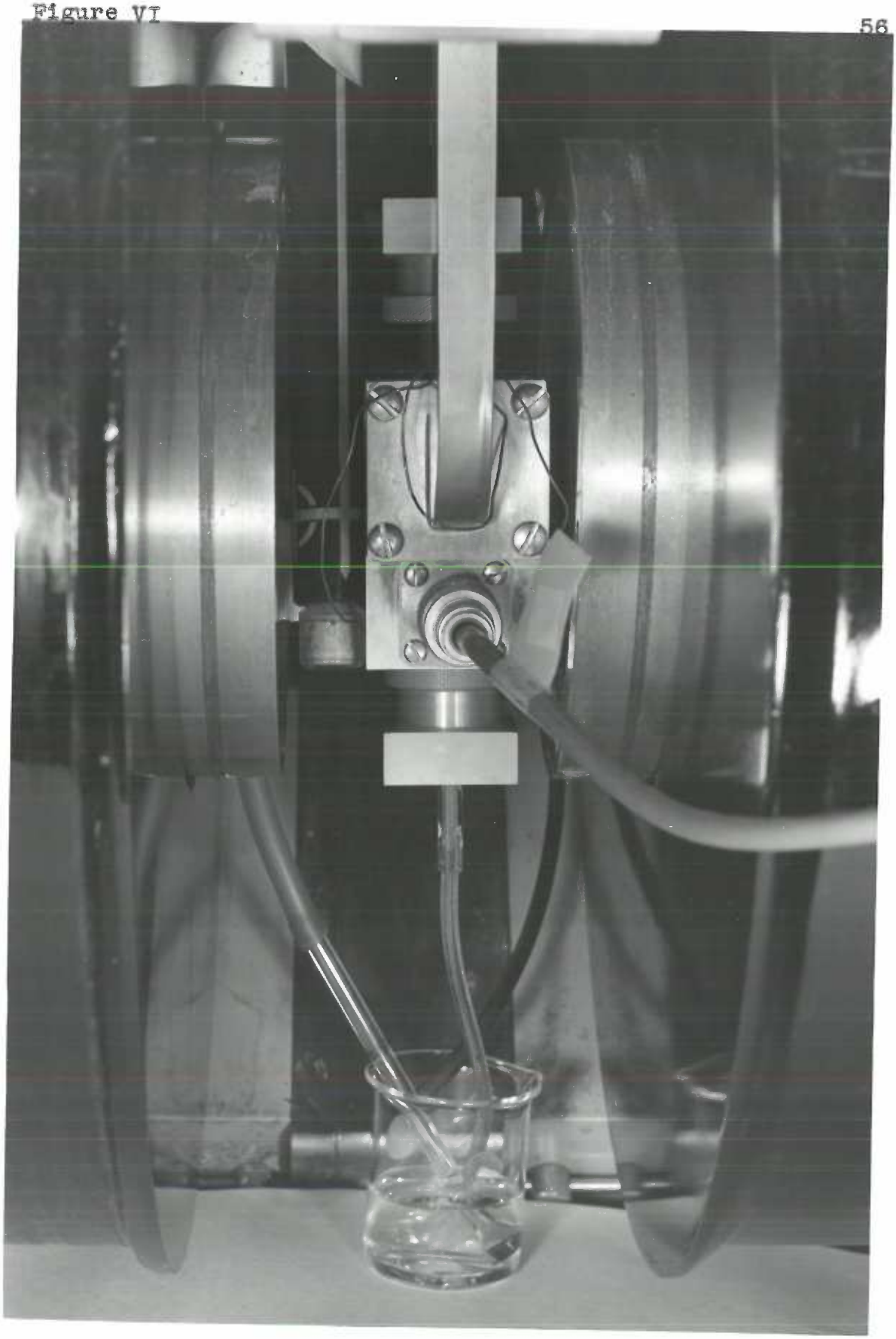


Figure VII The flat quartz cell with intake capillary for the recirculation apparatus attached.

Figure VII

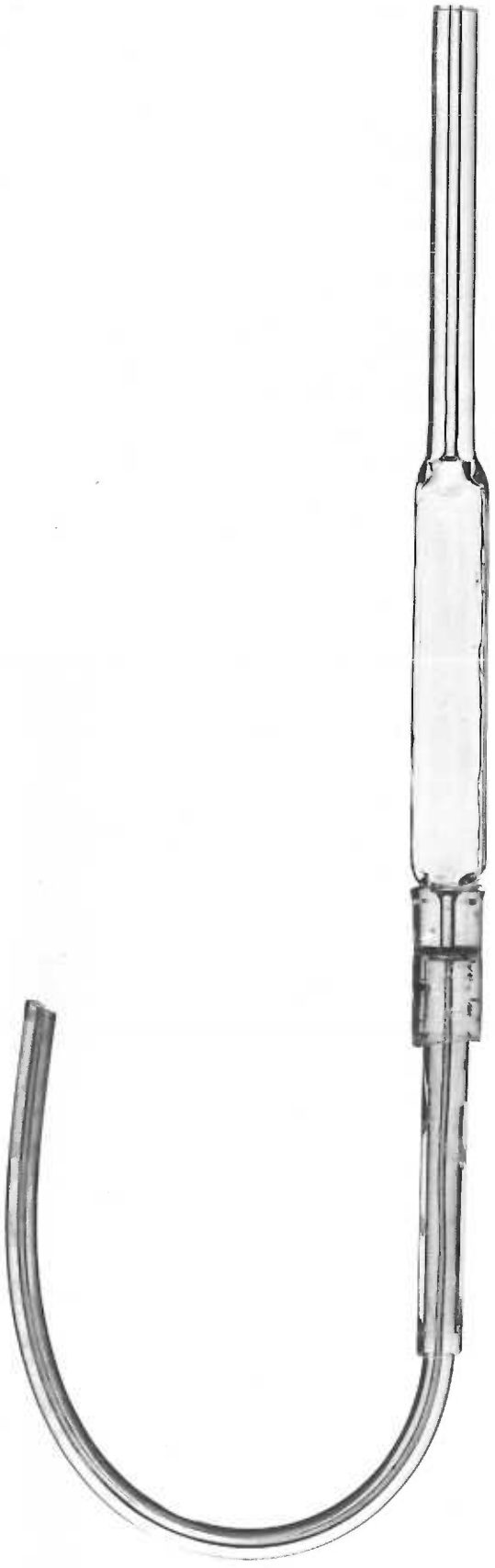


Figure VIII The recirculation apparatus in the Cary recording spectrophotometer. The cover to the sample compartment is replaced with a black wooden piece with a hole in the center. The opening admits the intake and delivery tubes of the Sigma pump, the gas bubbler, and the fine plastic capillary attached to the enzyme delivery syringe. The syringe is taped to the cover in such a way that when the assembly is covered with a black cloth to keep light out of the sample compartment the plunger can be depressed quickly without disturbing the set-up.

Figure VIII

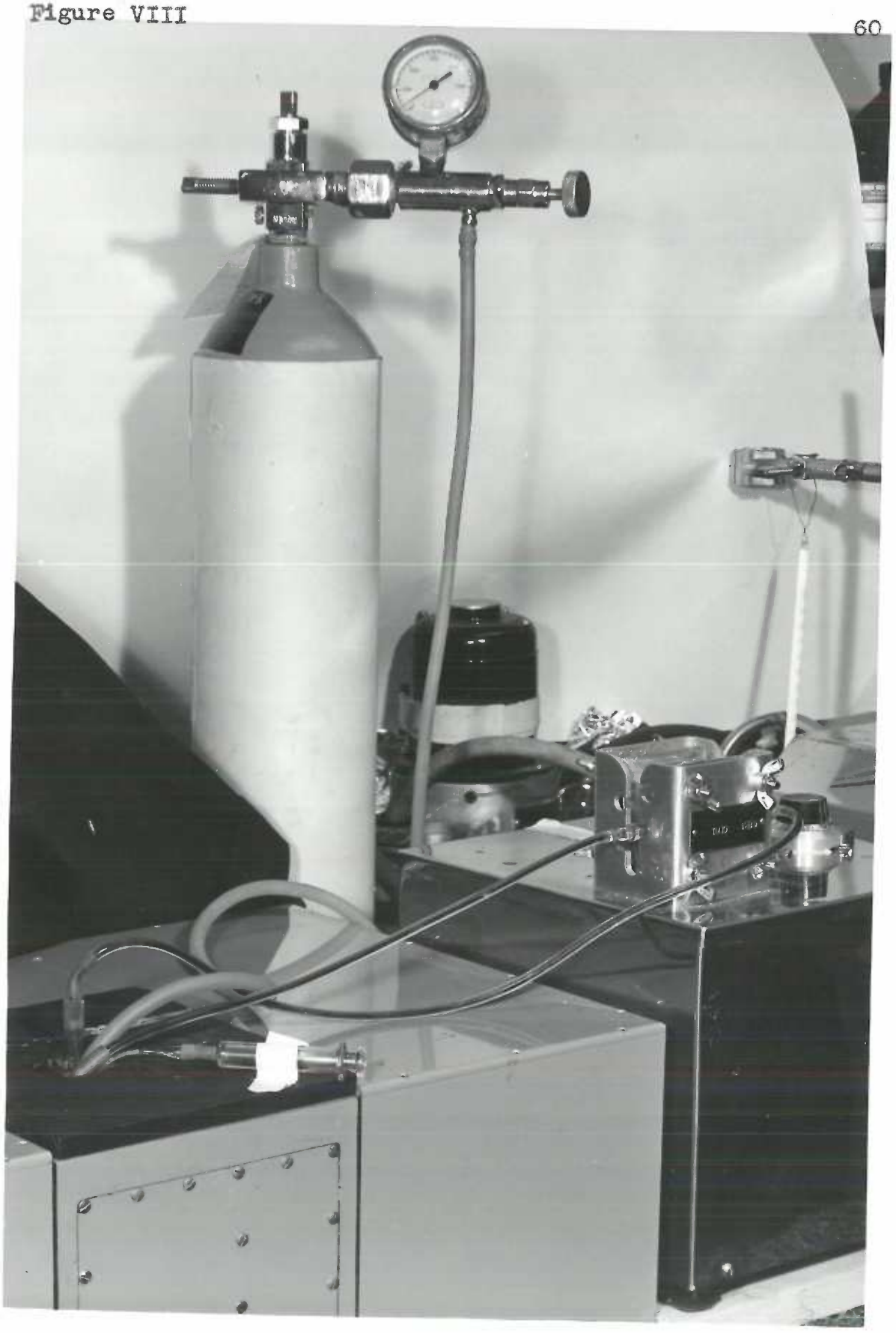


Figure IX Viewed horizontally, this figure shows the inside of the sample compartment of the spectrophotometer with the quartz cell mounted perpendicularly in the light path and still connected to the reservoir and recirculating system.

Figure IX

RECIRCULATION AND GAS EXCHANGE SYSTEM

The quartz cell described above was fitted with a plastic capillary tube on the lower end which dipped into a reservoir. The volume of solution from the end of the capillary to the middle of the cell was 0.2 ml. Buffer and catechol were mixed in the reservoir and enzyme added at zero time. The mixture was circulated by means of a Sigma pump at 1.4 ml. per second. Gas was bubbled rapidly into the reservoir through a fritted glass disc. A small amount of DuPont anti-foam was added to keep the reaction mixture from frothing. After each run the system was washed out thoroughly with deionized water and buffer. The hold-up in the pumping system was 4 ml.

OPTICAL MEASUREMENTS

The quinone concentration in the system was followed with a Cary recording spectrophotometer, Model 14, set at 390 m . The quartz cell was fixed in the sample compartment by means of shim blocks and tape so that it was held firmly in the light path and not moved when samples were changed, etc. The same recirculation set-up was used as for the e.p.r. experiments.

ENZYME PREPARATION

The preparative procedure used was a modified version of that published by Frieden and Ottesen (21). They adopted the first portion of the method of Kertesz and

Zito (34), adding the DEAE-cellulose column of Brown and Ward (7). We used commercially grown mushrooms supplied by Pioneer Fruit Company who usually received them from the grower late in the day, shipping them to us the following morning. We immediately placed them in the freezer at -72°C and stored them there for varying periods of time. We found that after a week or more their tyrosinase activity gradually began to drop. We did not obtain any high activity preparations from mushrooms stored a month or more. Those stored on the bottom of the freezer where they were not disturbed or exposed to the air kept better than those near the top. In time they became quite dark in color.

The mushrooms, two pounds at a time, were made into an acetone powder in a Waring blendor with approximately two liters acetone which was at -72°. The acetone was removed on a large Buchner funnel while the filter cake was kept cold by dry ice placed on the rubber dental dam used to keep air away from the cake. The process was repeated, grinding more finely the second time. The filter cake was returned to the blendor and homogenized at low speed for 2 or 3 minutes with two liters 30% acetone which had been cooled to 0°C. This mixture was divided into centrifuge bottles and centrifuged at -3°C for 10 minutes at 7000-8000 r.p.m. Allowing time for the centrifuge to attain this speed at this load, and then slow down, this step takes about an hour. It

should be accomplished as quickly as possible. The supernatant was measured and placed in the freezer. Two volumes of freezer temperature acetone were added quickly with stirring. The precipitate was allowed to settle for an hour or two, no longer, in the freezer. It was then collected in a Szent-Gyorgyi-Blum continuous flow, 8-tube, centrifuge apparatus at -20°C. The feed reservoir was placed in an outer container of dry ice until the solution was all drawn into the centrifuge. Internal cooling with dry ice cannot be used with a siphon feed system. The precipitate was washed with cold 75% acetone and then dissolved in the least amount of water. Ten ml. per tube, with 2 10 ml. washes poured from tube to tube, made about 100 ml. of solution and was usually found to be sufficient. The protein solution may be stored frozen at this point.

The above solution was made 1% in Calcium acetate by addition of cold 10% solution with stirring. It was frozen overnight, thawed and centrifuged. The precipitate was discarded. The solution was then made 60% saturated in ammonium sulfate by the addition of solid material. It was allowed to stand in the refrigerator an hour or two and centrifuged. The precipitated protein was dissolved in the least volume of deionized water. Cold, saturated ammonium sulfate solution was added to 30% saturation. Any precipitate which formed at this point was centrifuged off and tested for activity. If it was active it was further fractionated with ammonium

sulfate, if not, it was discarded. The main portion of the solution was then made 50% saturated in ammonium sulfate. The precipitate contained the bulk of the activity. It was centrifuged, washed twice with 50% saturated $(NH_4)_2SO_4$ and dissolved in the least volume of deionized water. It was dialzyed overnight against 2 liters of .01 M phosphate buffer, pH 8.

About 50 g. of standard grade (Peterson and Sober) DEAE cellulose (64) was washed with deionized water and 0.1 M EDTA solution. It was then stirred with .01 M phosphate buffer, pH 8, and packed quite tightly, taking care to avoid air bubbles, into a 2.5 cm. column to a height of about 30 cm. The column was equilibrated with .01 M. phosphate buffer, pH 8, and set up on a fractionator in the cold room. The flow rate should be about 2 ml./minute. The dialyzed, assayed enzyme solution described above was placed on the column with care, to be sure that the colored material did not go farther down than 1/3 of its length. Additional buffer was used to distribute the material properly and make sure the column was not overloaded and that the effluent was colorless. The flow rate was reduced somewhat by the colored material.

Elution of an inactive protein as described by Frieden and Ottesen (21) and Frieden and Karkhanis (31) was brought about by washing with .04 M phosphate buffer, pH 8. When the optical density at 280 m of the effluent was below 0.1, the elution of the enzyme with

.08 M phosphate buffer was carried out. Additional activity was eluted with 0.1 M buffer. It is possible that the efficiency of the elution of the enzyme could be increased by a gradient elution technique.

The tyrosinase activity obtained at this point was up to 25% of that found in the first acetone precipitate. If the protein was determined by absorption at 280 m according to the method used by Frieden, and the activity by Miller-Dawson technique, the specific activity was 1000 units/mg.

This preparation was used directly in the kinetic experiments.

Since a very large quantity of tyrosinase was needed for these and further experiments, a modification of the preparative procedure was attempted for the purpose of increasing output. 300 pounds of mushrooms were made into acetone powder and stored in the freezer for one to four months. The powder was then extracted with 30% acetone in large batches. After precipitation with acetone and collection of the precipitate as described above, the aqueous solution was treated with calcium acetate and dialyzed as described by Frieden and Ottesen. It was carried through the other steps as described above.

It was discovered that:

1. The acetone powder turns dark on standing in the freezer. The oldest batches were very dark.

2. Extraction with 30% acetone gives a dark brown solution.
3. The acetone precipitate is dark brown and actually liquid in the bottom of the centrifuge tubes.
4. The activity of the aqueous solution is very low.
5. Extended dialysis produces a great deal of color both inside and outside the dialysis bags.
6. The more color in the preparation, the larger the column needed to take it out and the lower the enzyme yield from the column.

It is best to order mushrooms in 25 pound batches, using a continuous process technique to obtain large quantities over a short period of time. As Kertesz (34) suggests, the entire acetone treatment should be completed in one day. Even the concentrated, purified enzyme stored at -72°C is not stable indefinitely but will lose half its activity in about six months.

ENZYME ACTIVITY DETERMINATIONS

The procedure of Miller and Dawson (56, 14) was used for the assay of enzyme activity. An attempt was made to modify the method to exactly simulate the conditions in the e.p.r. cell. In 0.1 M phosphate buffer, at pH 7.6, the endpoint was difficult to see, but the activity was little different from that determined at the usual pH 5.1. However, when 50% and 100%

oxygen were bubbled through the solution instead of air, the oxidation rates increased by factors of 1.4 and 1.6, respectively.

CHEMICALS

Water: All the water used in this work was distilled and then deionized.

Buffers: The buffers at pH 7 and above were phosphate mixed from stock solutions as described in "Methods in Enzymology" (vol. I, p. 143). The buffer used in the Miller-Dawson assay procedure was McIlvaine's citrate-phosphate, pH 5.1. For the experiment in which tyrosinase and peroxidase were compared at pH 5.3, acetate-acetic acid buffer was used. All buffers were checked against Beckman standard buffer pH 7 on a Zeromatic pH meter.

Catechol: The catechol was resublimed under vacuum, m.p. 103°

4-Methylcatechol was recrystallized from benzene, m.p. 65-65.5°

3, 4-dihydroxyphenylacetic acid cyclohexylamine salt obtained from California Corporation for Biochemical Research, started to decompose at 185°, melted at 195-195.5°

4-isopropylcatechol, and 3, 5-diisopropyl catechol were obtained from Aldrich Chemical

Company and, although impure, were tested qualitatively as free-radical producing substrates for tyrosinase.

Peroxidase: Crystaline HRP kindly furnished by Dr. Yamazaki R.Z. = 3.0

O-nitrophenol: Eastman m.p. 45-45.5°

EXPERIMENTAL

FLOW EXPERIMENTS

In these experiments 1 liter of buffer, pH 7.6, was placed in each bottle of the flow apparatus. Nitrogen was bubbled into the left-hand bottle and oxygen into the right-hand bottle for an hour. Meanwhile, the e.p.r. spectrometer was warmed up and tuned to the cavity with the cell filled with buffer solution. Catechol, 2.2 g, was mixed into the left-hand bottle. To the right-hand bottle, enzyme was added and the stoppers of both bottles were quickly and tightly wired down. The pressure was increased a little and the flow started slowly, while the magnetic field was scanned to find and identify the signal. When the signal was located, the scan was stopped and the field adjusted to the highest peak of the derivative curve. The flow was then stopped. When a constant base line had been obtained, i.e. the free radical had decayed to such a small value that the signal was down in the noise and undetectable, the pressure was adjusted to the first of three predetermined levels and the flow started. The flow was then stopped suddenly and the course of the reaction recorded as a trace of free radical accumulation and decay. The runs were repeated at each carefully measured flow rate several times. Enzyme and catechol concentrations were varied and the findings are described in the "results" section. The above experimental work was repeated with

peroxidase as the enzyme instead of tyrosinase. The work was again repeated in 0.1 M acetate buffer at pH 5.3 with both enzymes. This work was published in March, 1961 (53).

RECIRCULATION EXPERIMENTS

The gas exchange apparatus was connected to the quartz e.p.r. cell as described in the Apparatus section. The cell was filled with buffer and the spectrometer tuned. A solution of catechol was placed in the reservoir and the pump was started. Upon the addition of enzyme from a syringe, the scan was started to search for the signal. The signal of o-benzosemiquinone has three peaks, well separated from each other and the overall signal is quite broad for a free radical (3). Signals for melanin have been reported as a single narrow line at g=2 (14, 52). Since there was a possibility that the semiquinone signal could decay into a melanin signal, and that the contribution of the melanin signal could not be separately distinguished, especially if the semiquinone signal were very weak because of low concentration of free radical, the magnetic field was adjusted to show the deflection in one of the derivative peaks of the higher g side peak. This, of course, gives lower sensitivity to concentration changes, but it eliminates the possibility of confusion with melanin signals. When the peak was found and the field adjusted for maximum deflection of the pen at that point, the

experiment was started.

To 10 ml. of .02 M catechol solution in the gas exchange reservoir was added 10 ml. of buffer, less the volume of enzyme solution. After gas, pump and recorder were on, the enzyme was added from a syringe as quickly as possible. The free radical at constant oxygen pressure was then traced as it accumulated and decayed. For the 50% and 100% oxygen runs both buffer and catechol solutions were equilibrated with these gases for at least an hour before the start of the experiment. Catechol decomposition was prevented in the case of the 100% oxygen by adding the solid to the oxygen saturated buffer just before the start of the experiment, and in the case of the 50% run dissolving it in nitrogen saturated buffer, using diluting buffer saturated with 100% oxygen. Enzyme and catechol concentrations were varied, with the results to be reported in the next section.

ATTEMPT TO FIND CUPRIC ION IN TYROSINASE

The tyrosinase from the column as used in the previous experiments was further purified by fractionation with ammonium sulfate and dialysis of the concentrated aqueous solution against 0.005 M disodium phosphate overnight. The protein concentration was 10 mg./ml., the activity 10,900 M-D units/ml., making the specific activity 1090 units/mg., and the molar concentration of enzyme (m.w. 100,000) 5×10^{-5}. If there are four copper atoms to the enzyme molecule, the copper ion was 2×10^{-4} M.

A small amount was placed in a 1mm. quartz e.p.r. tube and frozen with liquid nitrogen. A special Dewar flask was placed in the cavity of the e.p.r. spectrometer and filled with liquid nitrogen. The tube of frozen tyrosinase was placed in the Dewar and the magnetic field scanned a number of times with negative results. We also added about 1/5 of its volume of 1mM. H_2O to the tyrosinase solution, frozen as before, and looked again for a copper signal, but found none. In a previous experiment we had found that very impure tyrosinase does give a copper signal which does not change upon addition of catechol. The result is inconclusive and further work awaits the production of large amounts of pure enzyme.

FREE RADICALS FROM OTHER SUBSTRATES

Tyrosinase is known to use many dihydric phenols as substrates. In order to facilitate study of the kinetics of tyrosinase-catalyzed reactions, the free radicals of some of these compounds were produced by alkaline oxidation and their absorption patterns traced on the e.p.r. They all show characteristic hyperfine structure. Each substance was made into 0.01 M. solution in Na_2HPO_4-NaOH buffer, pH 11, and mixed with an equal volume of 0.01 M. potassium ferricyanide. A syringe was used to fill the flat quartz cell from the bottom. The free radicals were stable for ten minutes to half an hour, giving ample time to observe and record the signal.

Figure X The electron spin resonance absorption derivative trace for o-benzosemiquinone obtained by alkaline ferricyanide oxidation. The hyperfine structure shows the effect of two major equivalent protons and two minor ones. The g-value, or spectroscopic splitting factor is 2. This curve is the same as that obtained by Hoskins (27) upon alkaline autoxidation of catechol.

Machine settings:

Modulation amplitude	25
Response time	2 - 0.3 sec.
Gain	500
Scan speed setting	10 gauss/min. or 1.1 gauss/cm.
Frequency	9.495 Kmc.

Figure X

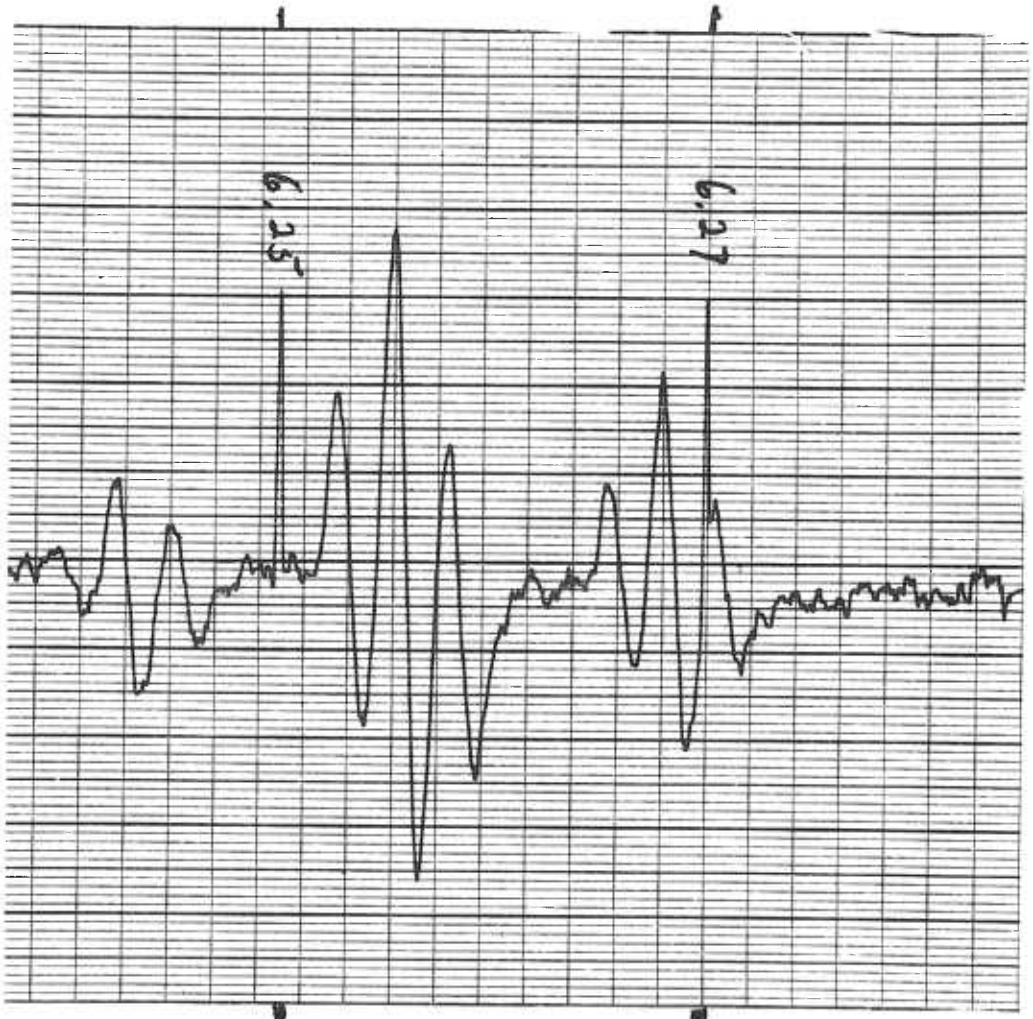


Figure XI Comparison of the e.p.r. signal of peroxylamine disulfonate, which was used to standardize the instrument, with that of o-benzosemiquinone. The areas under the absorption curves are proportional to the concentrations of free radicals in the solutions. The o-benzosemiquinone signal recorded in this figure was obtained by the oxidation of catechol catalyzed by tyrosinase at pH 7.6. Catechol concentration: .01 M; enzyme: 15 units/ml.; buffer: 0.1 M phosphate

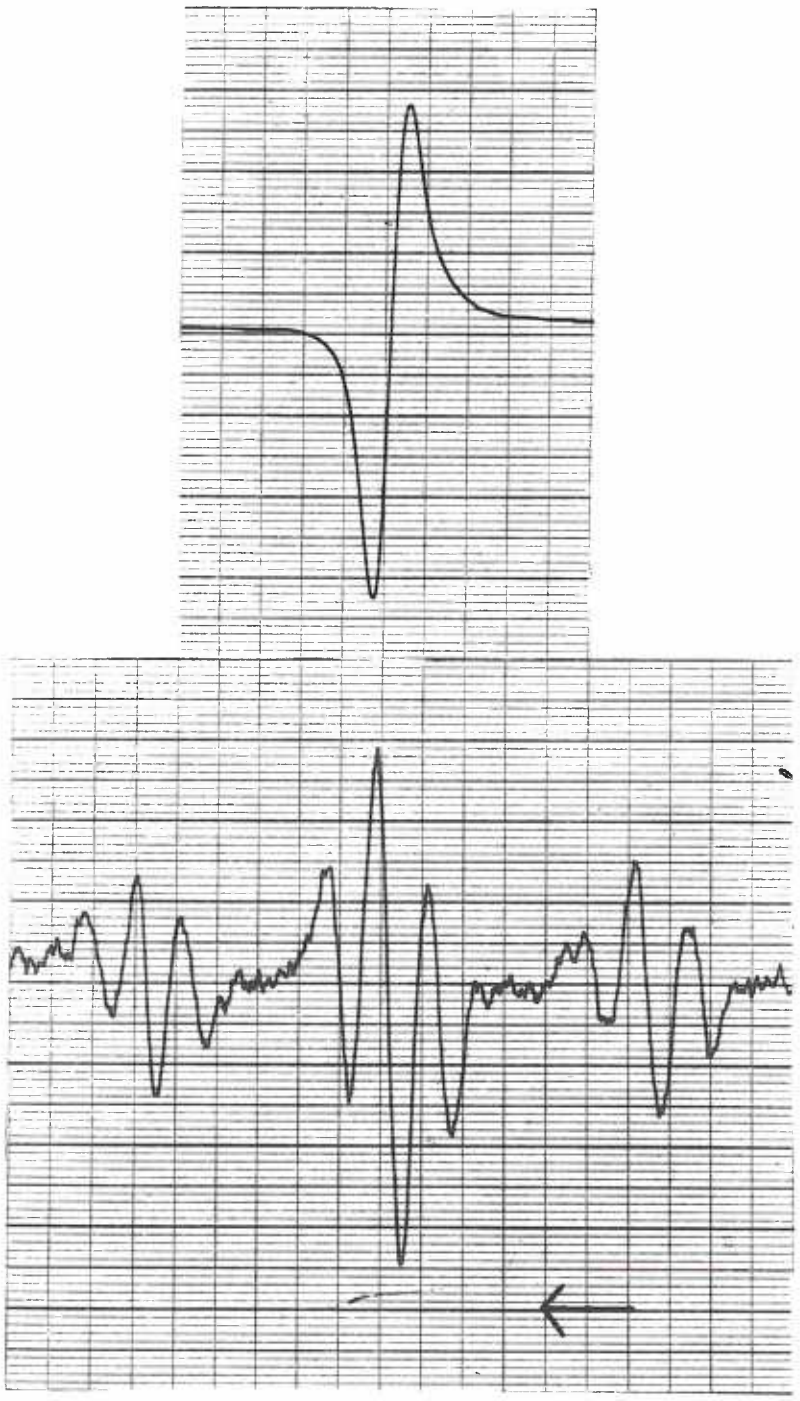
Concentration of peroxylamine disulfonate: $.244 \times 10^{-3}$ M

Machine settings:

Modulation amplitude	25
Response time	0.8 sec.
Gain	500 for o-benzosemiquinone
	2.5 for standard
Scan speed setting	10 gauss/ min. or 1.1 gauss/ cm.

The arrow shows direction of increasing magnetic field. The g-values of the two signals are not the same.

Figure XI



In comparing unknown and standard signals for determination of free radical concentration (28), it is necessary to make sure of the following conditions:

1. The filling factors for the two samples must be identical. The samples should both be either "line" or "point" samples, not one of each. The solvents should be the same, materials of high dielectric constant distort the rf field pattern.
2. The cavity must be matched to the klystron frequency to the same degree for each sample.
3. The crystal leakage must be adjusted to the same level.
4. The amplifier gain settings must be known.
5. The modulation amplitude should be the same for both samples.

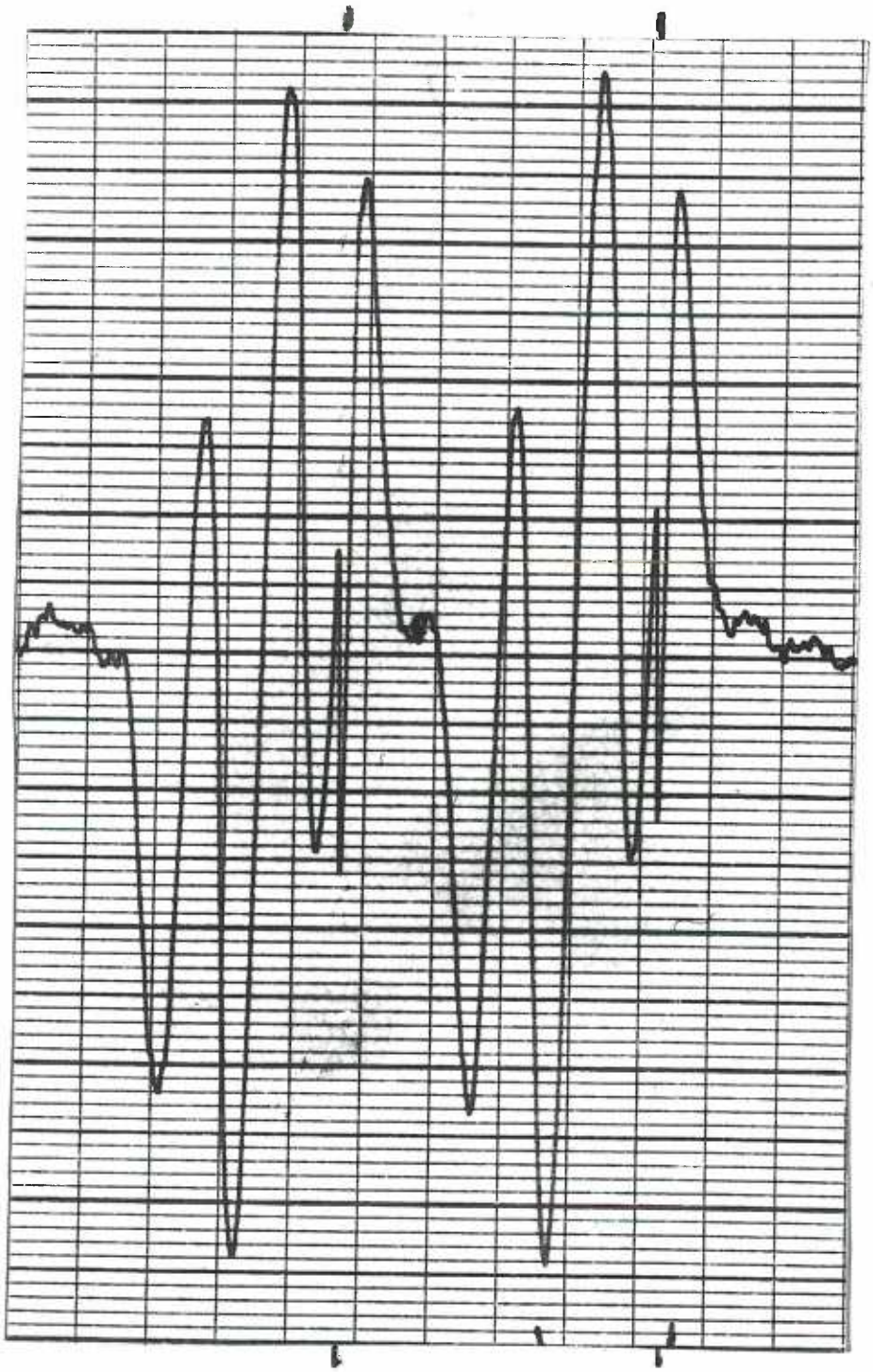
The above qualifications were met by using the same quartz cell for the standard as for the unknown, the same solvent, water, the same tuning procedure with the same power mode and figure on the oscilloscope for both, the crystal leakage set to a standard number, 300 micro-amps, the amplifier gains were recorded. It was found by Drs. Yamazaki and Narni that the variation of signal area is directly and linearly proportional to the gain setting. The modulation amplitude was 25 for both standard and sample.

Figure XII The electron spin resonance absorption derivative trace for .01 M 4-isopropyl catechol oxidized by .01 M potassium ferricyanide at pH 11. The main splitting gives a doublet which is further split into triplets. This indicates the influence of one main proton with two less important ones.

Machine settings:

Modulation amplitude	25
Response time	0.8 sec.
Gain	500
Scan speed setting	1.1 gauss/cm.
Klystron frequency	9.495 Kmc.

Figure XII



MAGNETIC FIELD IN GAUSS

Figure XIII The signal for the enzyme-generated free radical of 4-isopropyl catechol. The substrate was .01 M in 0.1 M phosphate buffer, pH 7.6.

Machine settings:

Modulation amplitude	50
Response time	0.8 sec.
Gain	500
Scan speed setting	2 gauss/cm.

Figure XIII

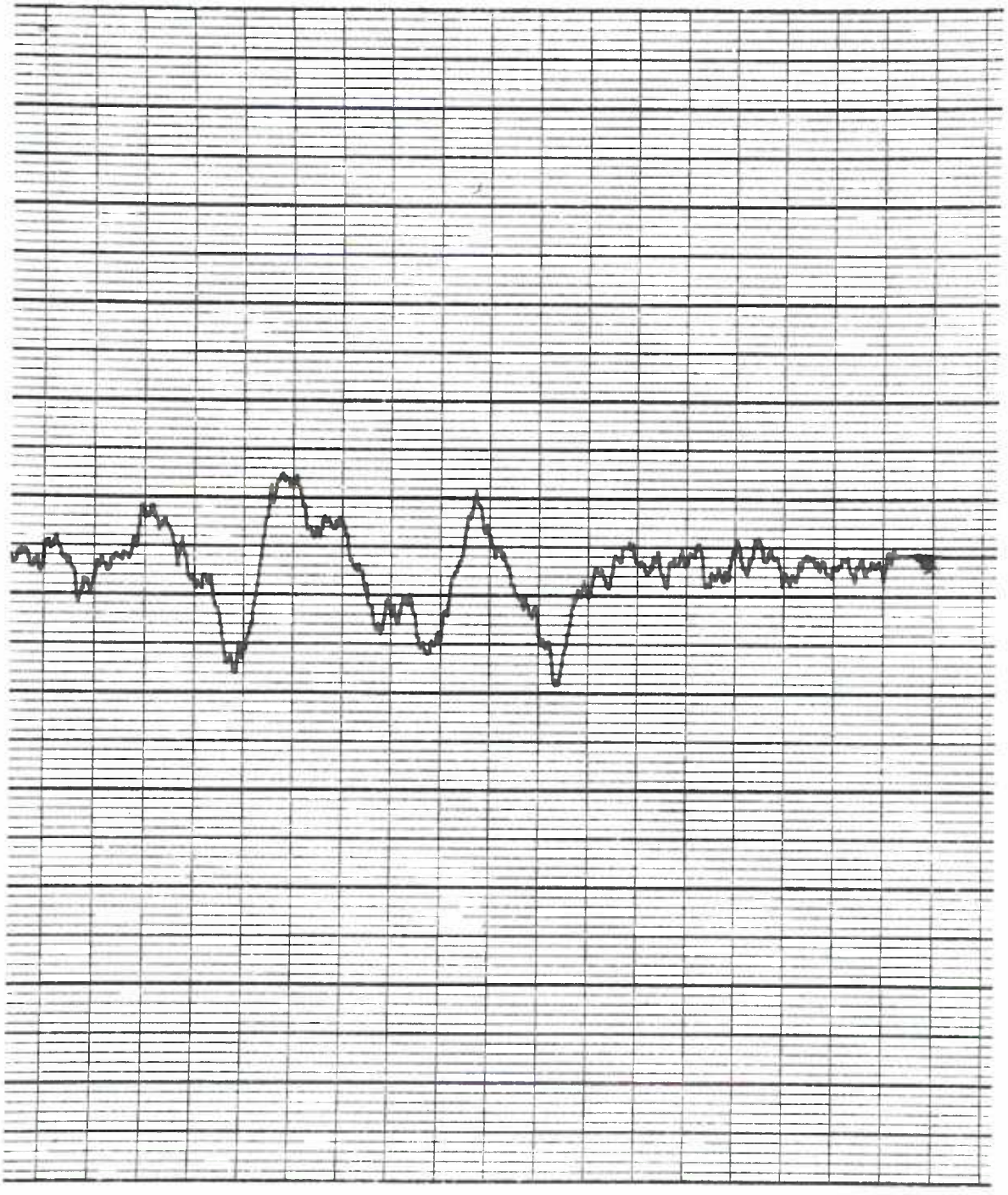


Figure XIV The electron spin resonance derivative trace of .01 M 3, 5-diisopropyl catechol oxidized by .01 M potassium ferricyanide at pH 11. The three main lines have unresolved finer structure. This indicates the influence of two main protons with some contribution from the isopropyl side chains.

Machine settings:

Modulation amplitude	25
Response time	0.8 sec.
Gain	500
Scan speed setting	1.1 gauss/cm.
Klystron frequency	9.495 Kmc.

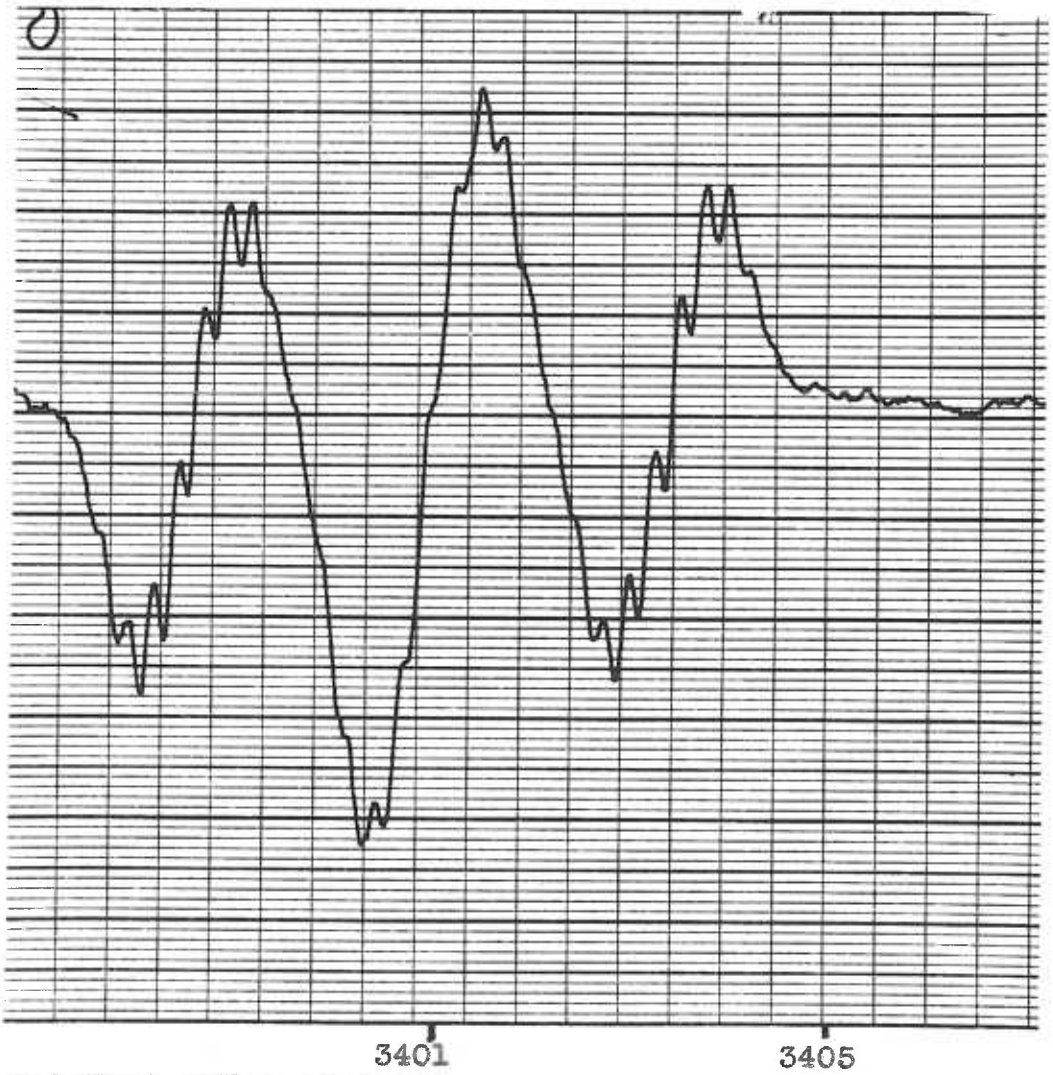

3401
3405
MAGNETIC FIELD IN GAUSS

Figure XV The same signal as Figure XIV generated from 3, 5-diisopropyl catechol by tyrosinase at pH 7.6.

Machine settings:

Modulation amplitude	25
Response time	0.8 sec.
Gain	1000
Scan speed setting	1.1 gauss/cm

Figure XV

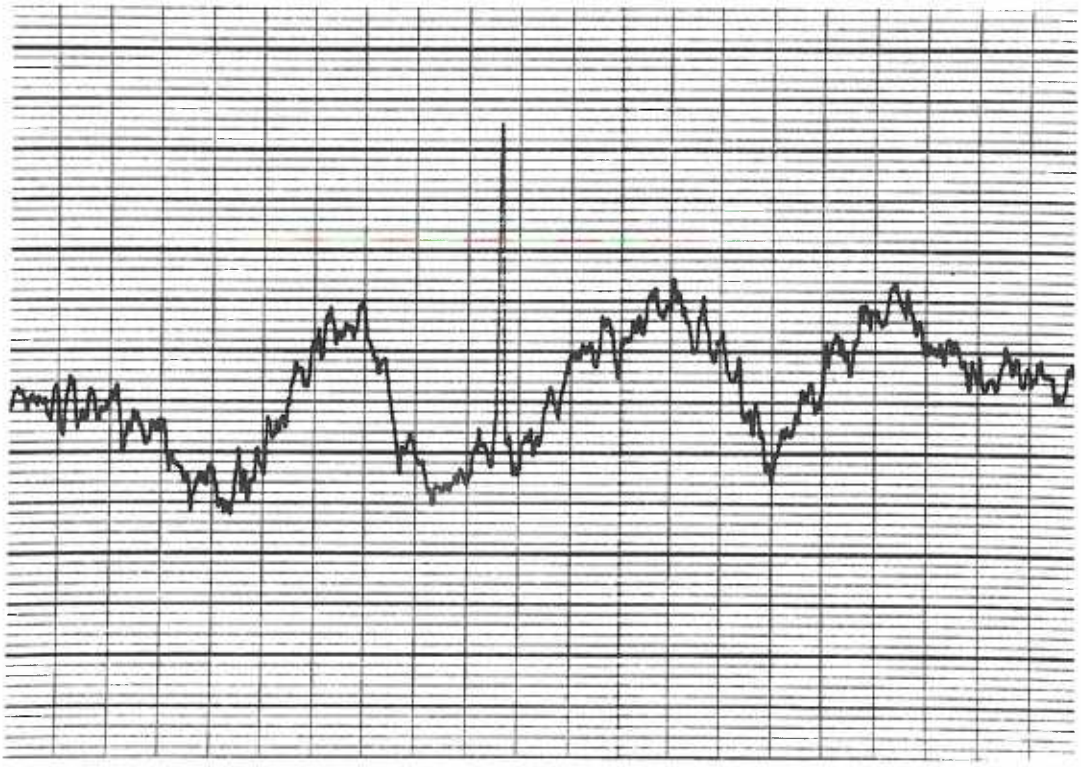


Figure XVI The electron spin resonance derivative curve for the semiquinone of .01 M 4-methyl catechol. The free radical is generated by alkaline oxidation. The hyperfine structure shows four main lines, indicating three equivalent protons. These are further split into triplets, which would indicate two protons of less influence. It is hard to rationalize this signal in terms of the structure of the radical. The insert shows the same signal, although weak, generated by oxidation in the presence of tyrosinase at pH 7.6.

Concentrations of reagents:

4-methyl catechol:	.01 M
enzyme	7 units/ml.
phosphate buffer	0.1 M

Machine settings:

Modulation amplitude	25
Response time	0.8 sec.
Gain	800
Scan speed setting	1.1 gauss/cm.

Figure XVI

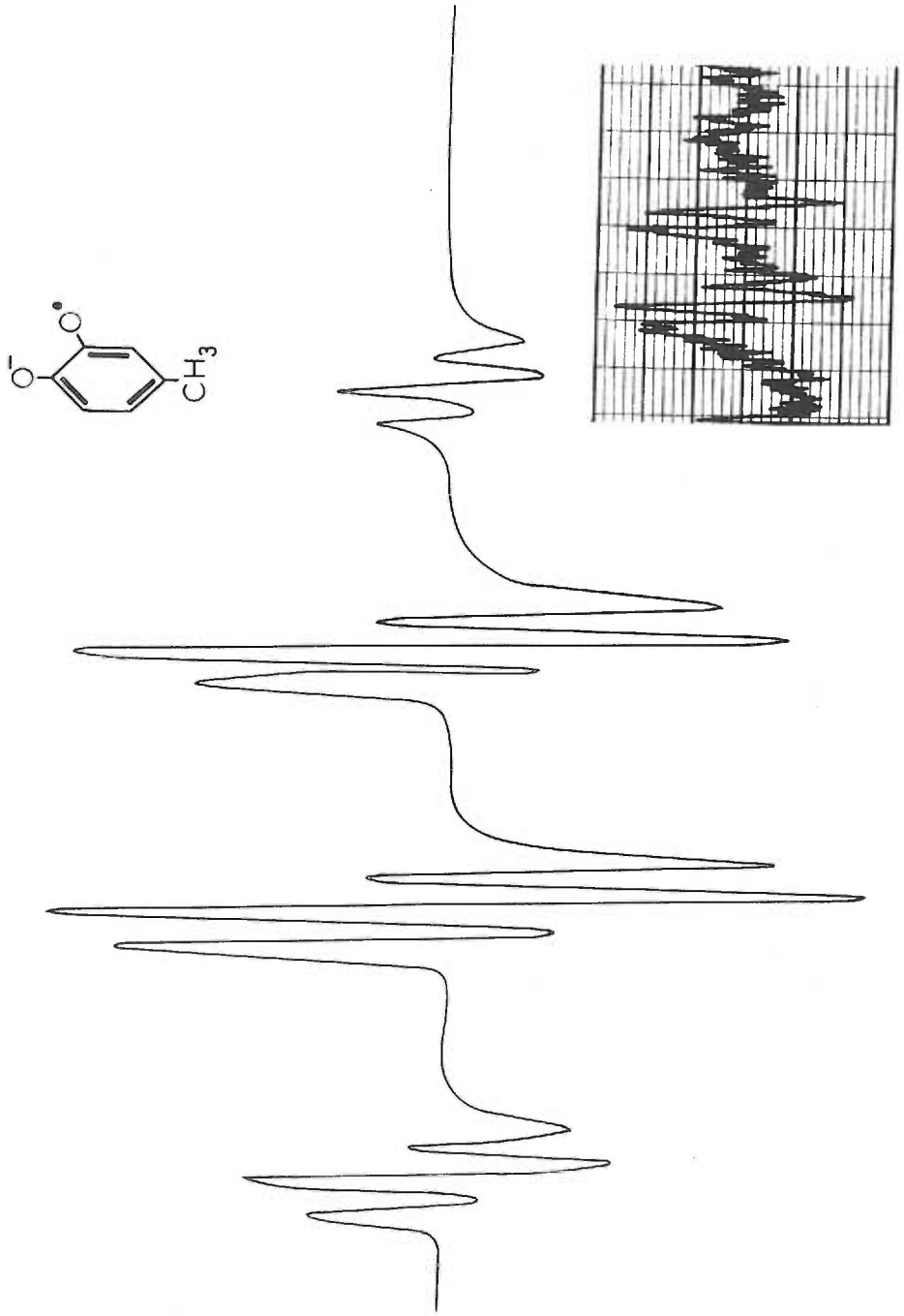


Figure XVII The electron spin resonance derivative curve for .01 M 3, 4-dihydroxyphenylacetic acid, in the form of the cyclohexylamine salt. The signal was obtained by alkaline oxidation. There are four main lines, indicating the influence of three protons. The splitting into doublets would point to the effect of a single proton.

Machine settings:

Modulation amplitude	50
Response time	0.8 sec.
Gain	800
Scan speed setting	1.1 gauss/cm.

Figure XVII

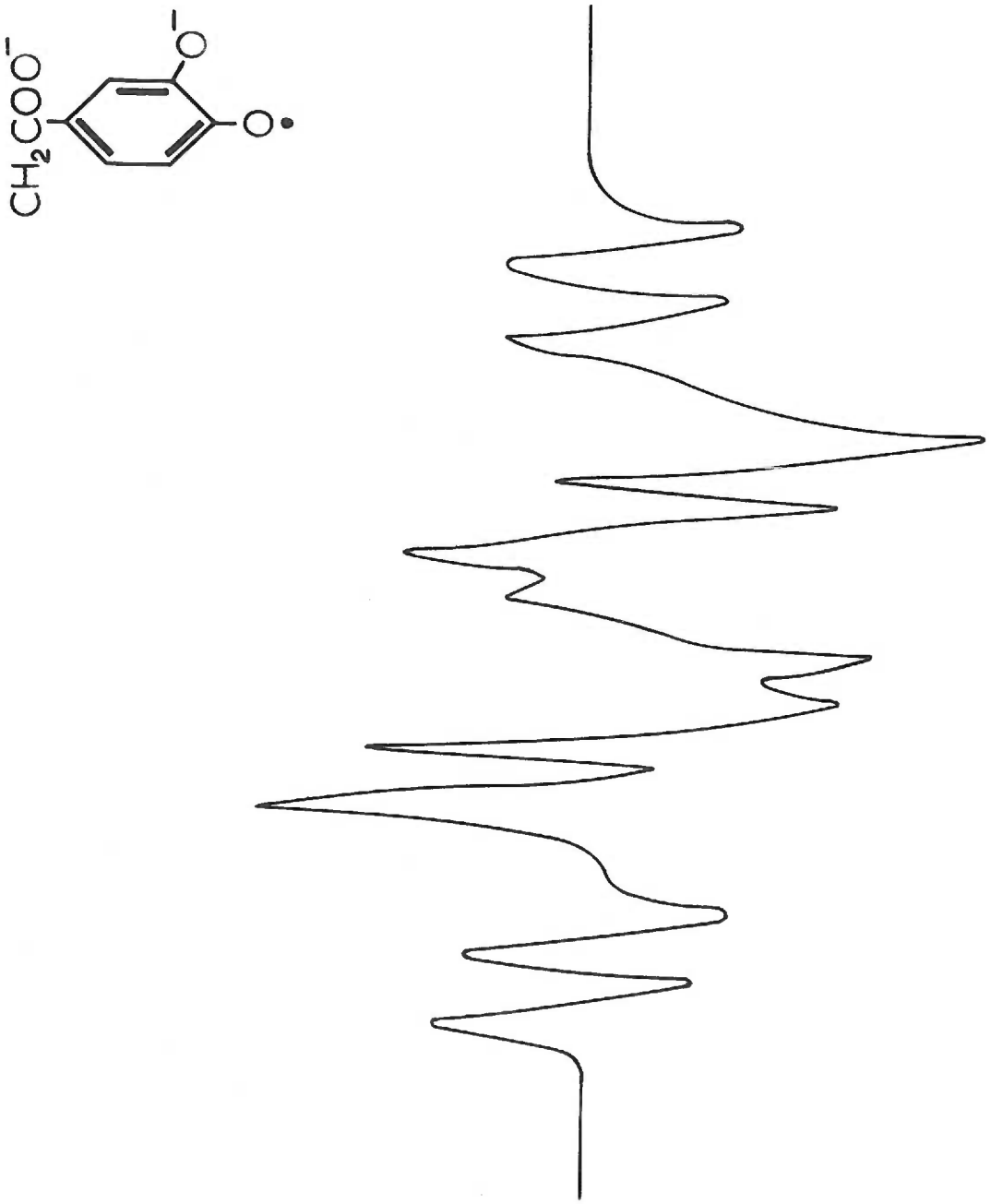
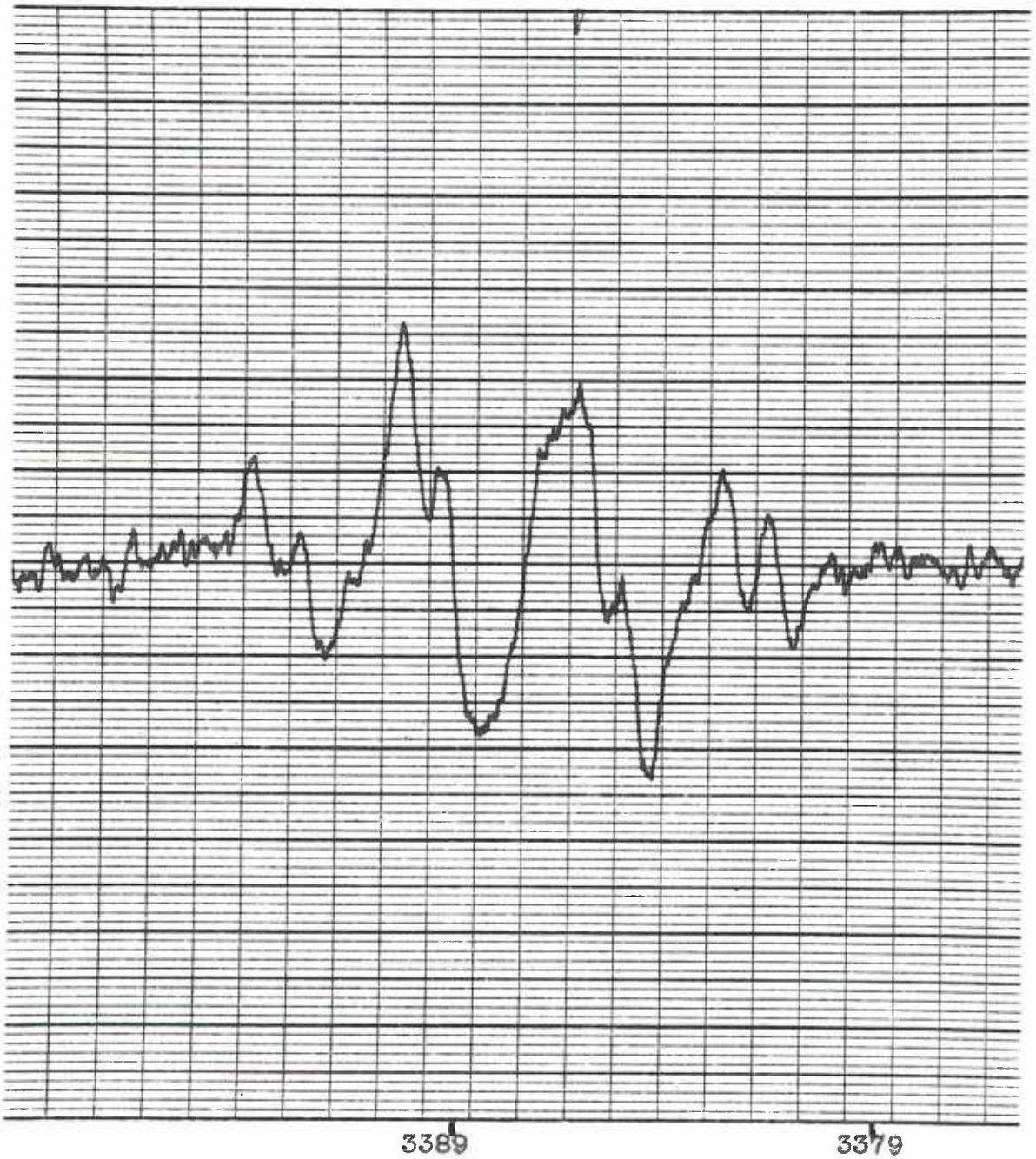


Figure XVIII Enzyme generated free radical of 3, 4-dihydroxyphenylacetic acid, cyclohexylamine salt.
Machine settings as in Figure XVII, except for scan speed which was 2 gauss/cm.
Klystron frequency 9.500 Kmc.

Figure XVIII



MAGNETIC FIELD IN GAUSS

These signals were also generated by oxidation of the catechols in the presence of tyrosinase in the flow apparatus. The signals are compared in Figures X to XVIII.

SPECTROPHOTOMETRIC EXPERIMENTS

The gas exchange apparatus was set up in the sample compartment of the spectrophotometer as described above. It was first necessary to determine the length of the optical path of the quartz e.p.r. cell. This was done by measuring the optical density of a solution at known concentration of a pure compound of known molecular extinction coefficient. Since the e.p.r. cell was not made for optical measurements it is neither optically flat nor uniform. O-nitrophenol was selected because it absorbs fairly near the wave length characteristic of o-benzoquinone, because the pure compound was available, because it is stable in solution and because its molecular extinction coefficient is given (66) at 345-7 mμ. The ratio C/D = 4.42 is given to simplify calculation, where C is the concentration in mg./100 ml. and D is the optical density. Since these figures are for a path length of 1 cm. it is only necessary to take account of the inverse ratio between path length and optical density to determine the average path length through the part of the cell traversed by the beam of light.

Thus, the quartz cell was balanced against a 1cm. quartz spectrophotometer cell, both cells filled with alcohol. The alcoholic solution of 44.2 mg. o-nitrophenol per 100 ml. was then pumped into the flat cell and the optical density determined. The measurement was repeated several times to make sure that it was reproducible.

The reference cell was filled with buffer, pH 7.6 and the recirculation system cleaned and filled with buffer. The zero adjustment was made at 390 mμ and the buffer in the reservoir discarded. The same kind of catechol solutions at different oxygen pressures were used as in the e.p.r. studies. This time the spectrophotometer was turned on and the enzyme introduced from a syringe which was fitted with a long, fine plastic tube dipping into the reservoir, but could be controlled from the outside of the sample compartment without permitting light to enter. The increase in optical density at 390 mμ was recorded as a function of time.

When some doubt arose as to whether the increase in optical density at 390 mμ was entirely due to the accumulation of o-benzoquinone the experiments were repeated with the spectrophotometer scanning back and forth from 360 to 480 mμ at the rate of 10 mμ/sec.

FLOW EXPERIMENTS

The results of the flow experiments are illustrated by the e.p.r. trace of Figure XIX. The free radical concentration in the center of the cell is determined by the time after mixing that it takes the solution to travel that far from the reservoir. The pen deflection rises quickly to the position corresponding to that free radical concentration. It remains constant until the flow is stopped. The free radical then accumulates in the reaction mixture in the cell as the oxidation progresses and begins to decay as the oxygen concentration drops. If the overall reaction rate is of the order of 2×10^{-4} M/sec. the oxygen is theoretically all used at 5.5 seconds because each mol of catechol oxidized requires ½ mol O_2. The time course of the reaction must not be linear after the oxygen concentration is reduced below the level corresponding to twice the 2 Km for oxygen at that catechol concentration about 10% at 0.01 M for prune enzyme. The oxidation does not stop suddenly, and free radical and quinone decay take over gradually rather than sharply at a certain point in the reaction. The shape of the curve and the value of the maximum is certainly affected by the exhaustion of oxygen and it is difficult to assess the effect of enzyme substrate concentration upon the free radical concentration under these conditions.

We found that enzyme concentration has little effect

upon the maximum free radical observed if the enzyme were above 25 units/ml., and that substrate concentration had a profound effect upon the maximum even though it was far above the Michaelis concentration for catechol.

Lissitzky et al (41) reported spectrophotometric evidence of free radical formation in tyrosinase catalyzed oxidation of catechol. They added tyrosinase to alcoholic catechol solutions at -10 to -20°C, froze the mixture in liquid nitrogen to a glass, and scanned its ultraviolet absorption spectrum. They found an absorption peak at 288 mμ which they attributed to o-benzosemiquinone, because it was the same as that obtained for irradiated catechol under similar conditions and it disappeared upon warming to room temperature. They did not specify pH. These conditions are so unusual for enzyme reactions that it is hard to make comparisons between this and other work.

The above experiments at pH 7.6 do not show whether the free radical is the first product of the tyrosinase-catalyzed oxidation of catechol or not. If, however, the height of the constant section above the base line of the above curves obtained during flow at various rates is carefully measured and plotted as free radical concentration against time after mixing, a smooth curve is obtained which represents the variation of free radical concentration with time during the very early

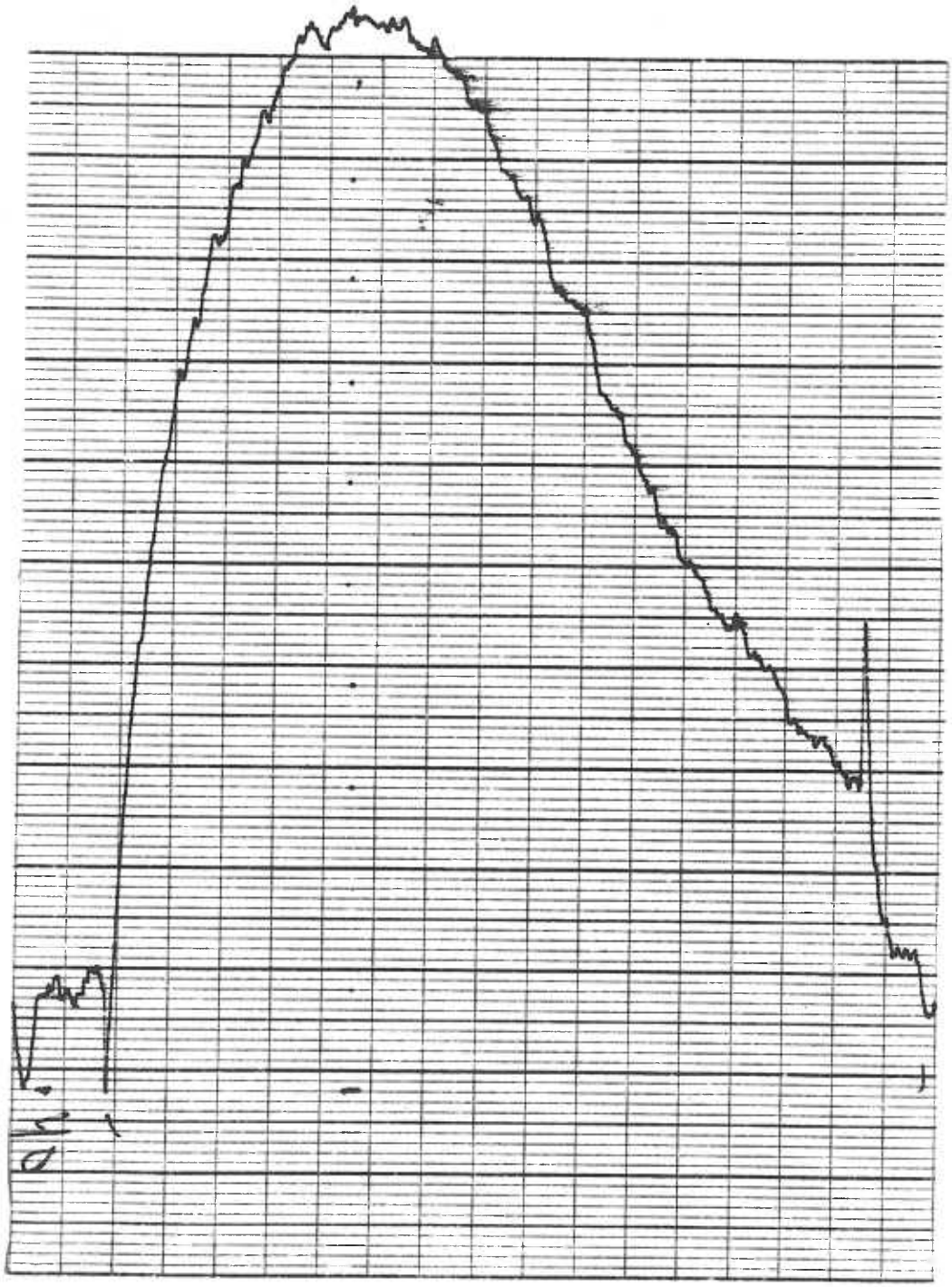
Figure XIX A typical e.p.r. spectrometer recording from the flow experiments. The first sharp dip on the left shows the start of the flow of reaction mixture through the cell in the cavity. The level of free radical detected rises and remains constant until flow is stopped at the next sharp dip. The reaction is followed through a maximum and a period of decay as shown and the sharp spike on the right marks the end of the experiment. The catechol concentration was .01 M, enzyme concentration, 1.9 units/ml. in 0.1 M phosphate buffer, pH 7.6. The flow rate is 8 ml./sec. The spectrometer is set on one of the high middle peaks of the o-benzosemiquinone signal. Each paper division represents 3 sec.

Machine settings:

Modulation amplitude	50
Time constant	0.8 sec.
Gain	500
Scan speed	5 gauss/cm.

The enzyme in this case is tyrosinase.

Figure XIX



of the reaction, before the oxygen concentration has been much depleted by the reaction. The catechol concentration is also virtually constant and typical free radical kinetics should describe the course of the reaction if free radicals are the first product and are produced faster than equilibrium by disproportionation can govern their concentration. The fact that the curve was smooth, not at all like the typical free radical curve indicated that either the free radicals were not produced fast enough to outrun the equilibrium concentration in which case enzyme concentration could be increased until this effect was produced, or that they were produced from the reverse reaction of quinone with catechol. In the latter case, o-benzoquinone would be the first product of the reaction. In order to decide this issue, it was necessary to produce o-benzosemiquinone at a rate comparable to the overall reaction rate catalyzed by tyrosinase to see if equilibrium is established so quickly that the free radical concentration could never exceed the equilibrium value.

In order to check this point, Dr. Isao Yamazaki kindly donated crystalline horseradish peroxidase. The same experiment, under the same conditions as with the tyrosinase, with the results plotted in the same way, completes the graph of Figure XXI showing the two o-benzosemiquinone curves of peroxidase, compared with the equilibrium curve of tyrosinase during the early stages of the oxidation at pH 7.6,

When peroxidase and hydrogen peroxide were permitted to oxidize catechol at pH 5.3, at a rate of 2.3 x 10^{-4}M/sec., a steady state concentration of semiquinone at 0.4 x 10^{-6}M was built up which decayed when the reaction velocity decreased. Tyrosinase, under the same conditions, acting at nearly the same overall rate, 2.6 x 10^{-4}M/sec., produced no detectable (i.e. less than 10^{-7}M, the lower limit for our instrument) free radical at pH 5.3.

Figure XX A typical e.p.r. spectrometer recording from the flow experiments with peroxidase at pH 7.6. The base line is shown at the left. At the first mark flow is started. It is stopped at the second mark and the increase and decay of o-benzosemiquinone in the reaction mixture is recorded as shown. A second experiment is shown in the same picture. The instrument settings are the same as in Figure XIX. The catechol concentration is .01 M, the peroxidase 1.68×10^{-2}M, hydrogen peroxide 1.2×10^{-3}M. The flow rate is 2 ml./sec.

Machine settings:

Modulation amplitude	50
Time constant	0.8
Gain	250
Scan speed	5 gauss/cm.

Figure XX

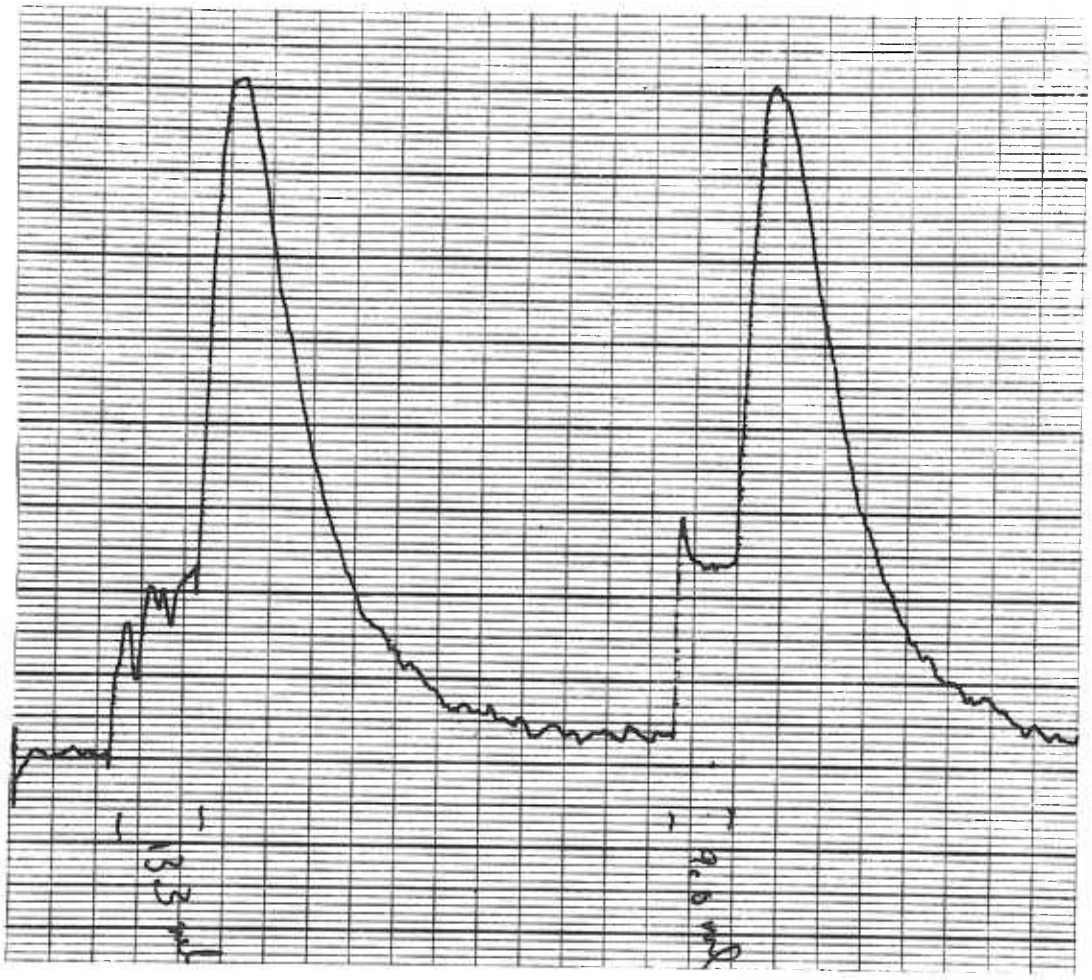


Figure XXI Comparison of o-benzosemiquinone generation by peroxidase and tyrosinase at pH 7.6, 0.1 M phosphate buffer, 0.01 M catechol, at 21°.

A: peroxidase ($v = 3.2 \times 10^{-4}$ M/sec.)

C: peroxidase ($v = 0.8 \times 10^{-4}$ M/sec.)

B: tyrosinase ($v = 2.2 \times 10^{-4}$ M/sec.)

Figure XXI

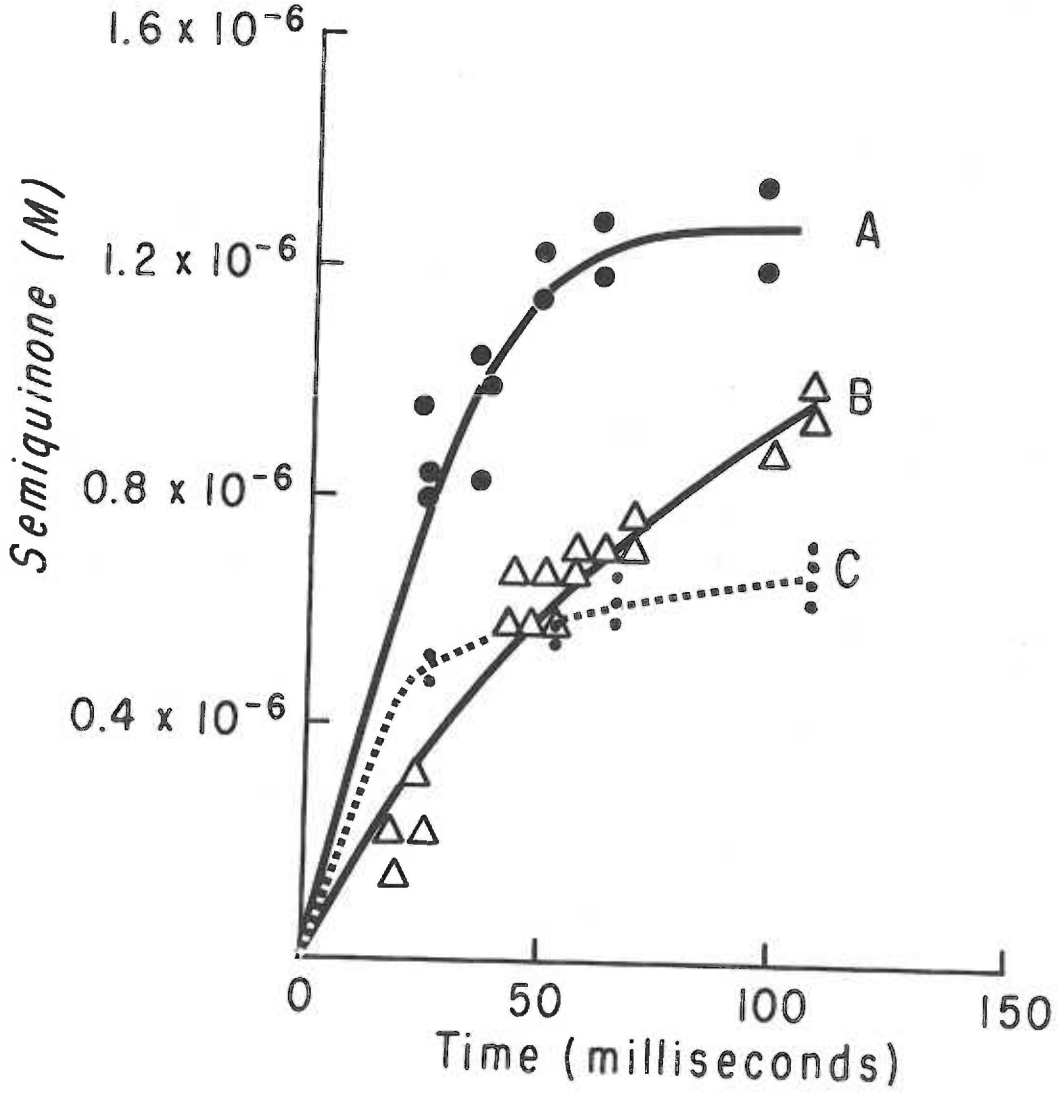

1.6 x 10⁻⁶
1.2 x 10⁻⁶
0.8 x 10⁻⁶
0.4 x 10⁻⁶
Semiquinone (M)
A
B
C
0
50
100
150
Time (milliseconds)

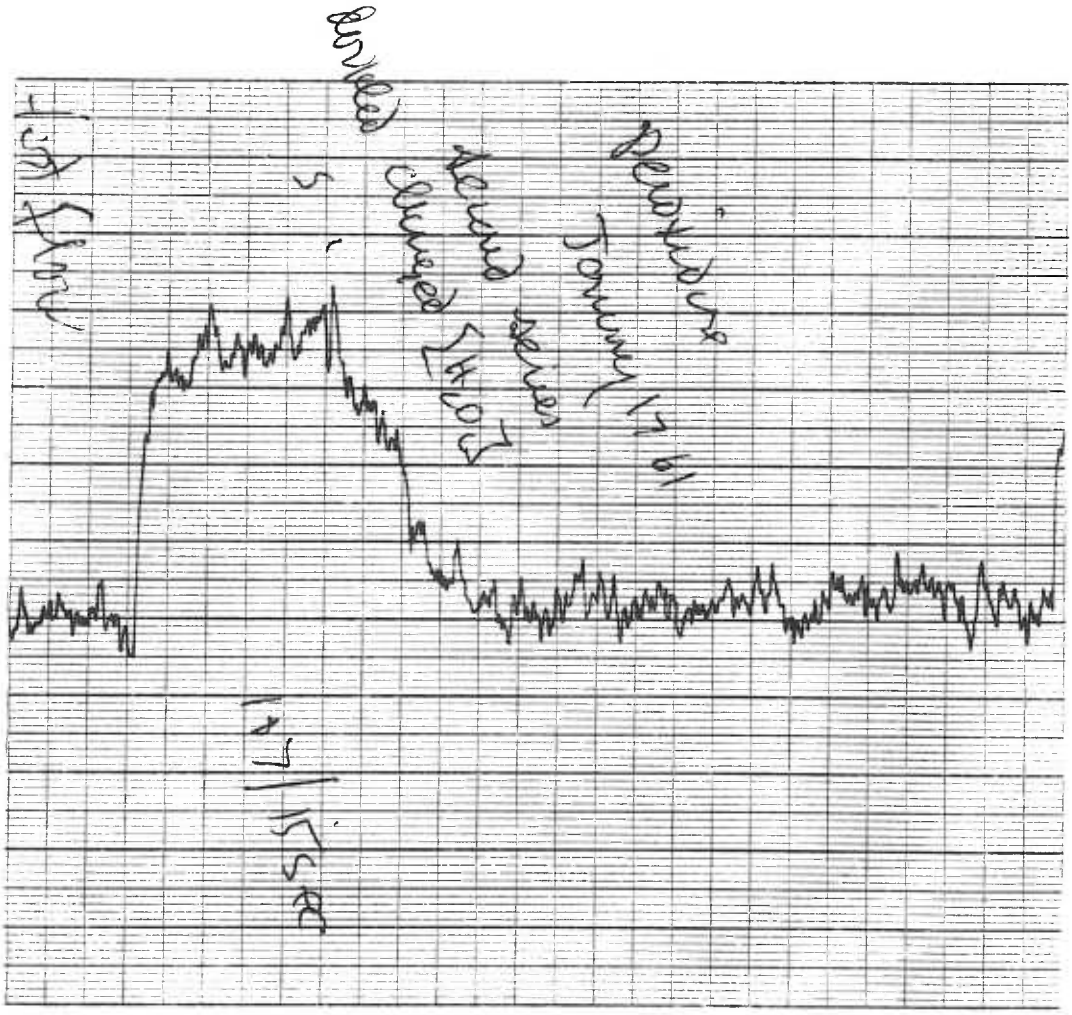
Figure XXII The e.p.r. spectrometer recording of the experiment with peroxidase at pH 5.3. The rate of flow is high in this case and the level of free radical during flow, shown at the left edge of the trace, is hardly above the noise of the instrument. When the flow is stopped the free radical concentration rises to a steady state, which shows decay only when the hydrogen peroxide becomes limited.

Catechol concentration, .01 M; enzyme concentration, 1.25×10^{-1} M; hydrogen peroxide, 1.2×10^{-3} M; 0.1 M acetate buffer.

Instrument settings the same as in previous figures:

Modulation amplitude	50
Time constant	0.8
Gain	1000
Scan speed	5 gauss/cm.

Figure XXII



RECIRCULATION EXPERIMENTS

In these experiments the oxygen concentration was held constant by recirculation of the reaction mixture through a gas exchanger. Thus the time course of the oxidation of catechol to quinone should be reflected by the concentration of free radical produced in the system. It should be possible to determine the equilibrium constant of the dismutation reaction if the quinone concentration could be determined independently. The free radical curves obtained are illustrated in Figures XXIII and XXIV. The maximum free radical concentration was determined by the oxygen and the catechol concentrations, and, at high oxygen concentration, the enzyme concentration also. The rate of free radical production was proportional to the enzyme, oxygen and catechol concentrations also. When quinone concentration was followed spectrophotometrically under the same conditions, it was found that its rate of accumulation was greatly increased by increased oxygen concentration at constant enzyme and substrate concentration (Fig. XXV). Change in catechol concentration should not have changed the rate of the reaction, but it does show an effect upon quinone accumulation. Increase in enzyme concentration at constant oxygen and catechol concentration has little effect upon the rate of quinone accumulation except at 100% oxygen saturation (see Fig. XXVI).

Under air saturation conditions, it was found that the rate of quinone accumulation doubled when the

Figure XXIII The free radical curve as traced by the electron spin resonance spectrometer. The solid line represents the data obtained with 28.8 units of enzyme/ml. The line with long and short dashes represents 14.4 units/ml. The dashed line shows 7.2 units/ml. The ordinate is calibrated in terms of molar free radical concentration by the calculation mentioned in the text.

A: 20% oxygen, .00835 M catechol

B: 20% oxygen, .0167 M catechol

C: 50% oxygen, .00835 M catechol

Figure XXIII

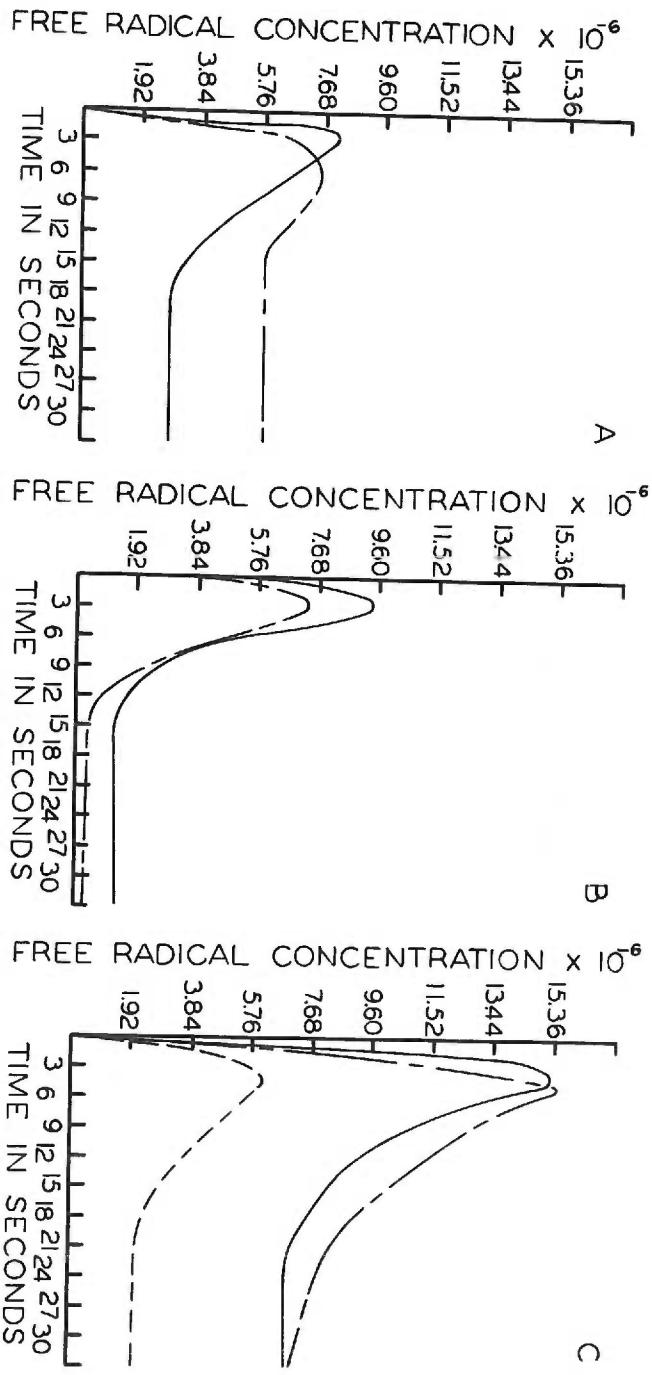


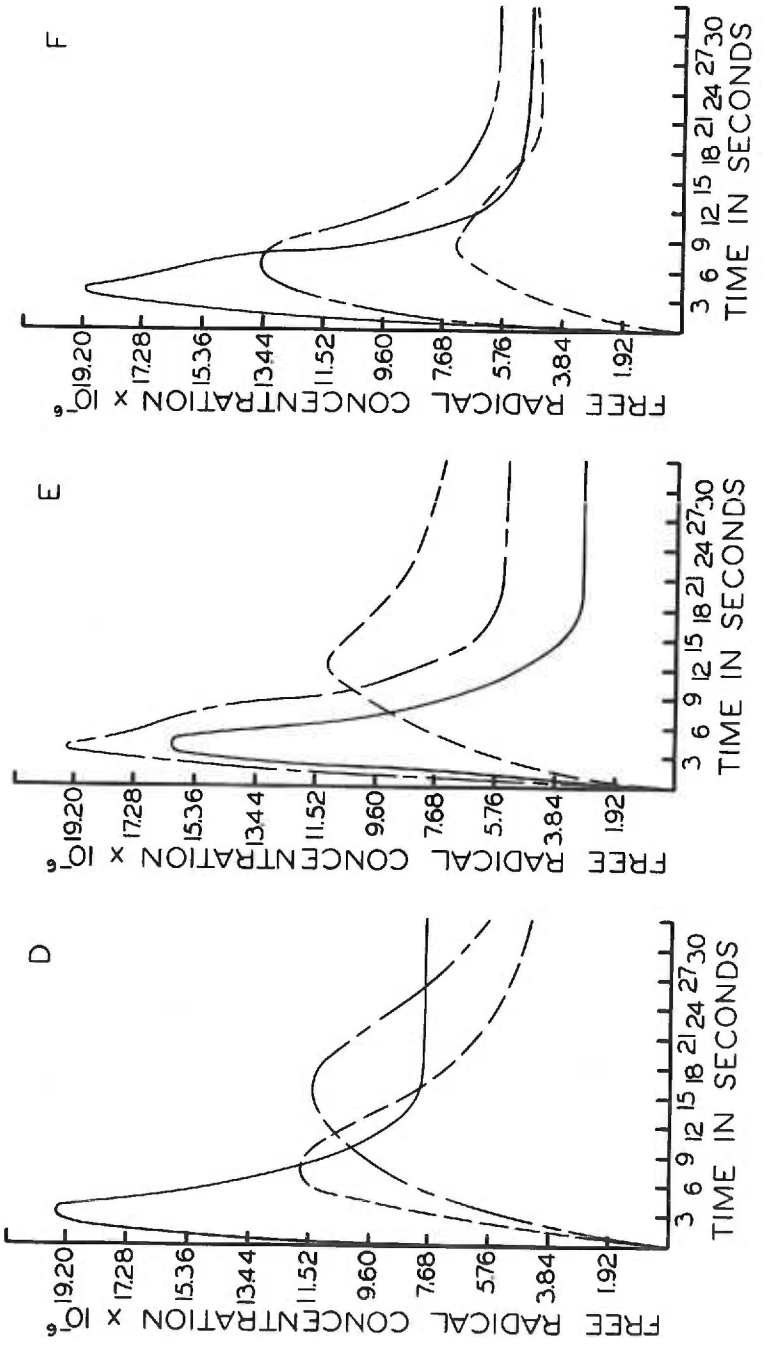
Figure XXIV Continuation of Figure XXIII.

D shows free radical at 50% oxygen, .0167 M catechol

E: 100% oxygen, .00835 M catechol

F: 100% oxygen, .0167 M catechol

Figure XXIV


D
E
F
FREE RADICAL CONCENTRATION x 10⁶
19.20
17.28
15.36
13.44
11.52
9.60
7.68
5.76
3.84
1.92
3 6 9 12 15 18 21 24 27 30
TIME IN SECONDS

catechol concentration doubled (Fig. XXVI). Enzyme concentration at constant catechol concentration has little effect. When the reaction mixture was saturated with 50% oxygen-50% nitrogen mixture, at .00835 M catechol, the quinone accumulation rate nearly doubled when the enzyme concentration was doubled from 7.2 units/ml. to 14.4 units/ml. However, when the enzyme concentration was doubled again, to 28.8 units/ml. there was no further change in rate. When the catechol concentration was increased to .0167 M, the rate for 7.2 units of enzyme/ml. was doubled over its value for half the catechol concentration, but doubling the enzyme concentration at this catechol concentration produced only a 25% increase in rate and then doubling the enzyme again produced only a small (4%) rise in quinone accumulation rate. When the reaction mixture was kept saturated with 100% oxygen, the rate for 7.2 units/ml. of enzyme was more than double that for 50% oxygen. Doubling the enzyme concentration at constant catechol concentration increased the rate about 60% and doubling the enzyme again brought about another 10% increase (Fig. XXVI). Doubling the catechol concentration gave a somewhat lower rate at 14.4 units/ml. of enzyme, but at 28.8 units/ml. the rate was increased again about 30%.

Plotting the free radical data upon the same scale as the quinone accumulation data permits comparison of the changes in these components with enzyme, oxygen

Figure XXV Velocity of quinone accumulation plotted against enzyme concentration at different oxygen concentrations.

A: .00835 M catechol

B: .0167 M catechol

1: 20% oxygen

2: 50% oxygen

3: 100% oxygen concentration

Figure XXV

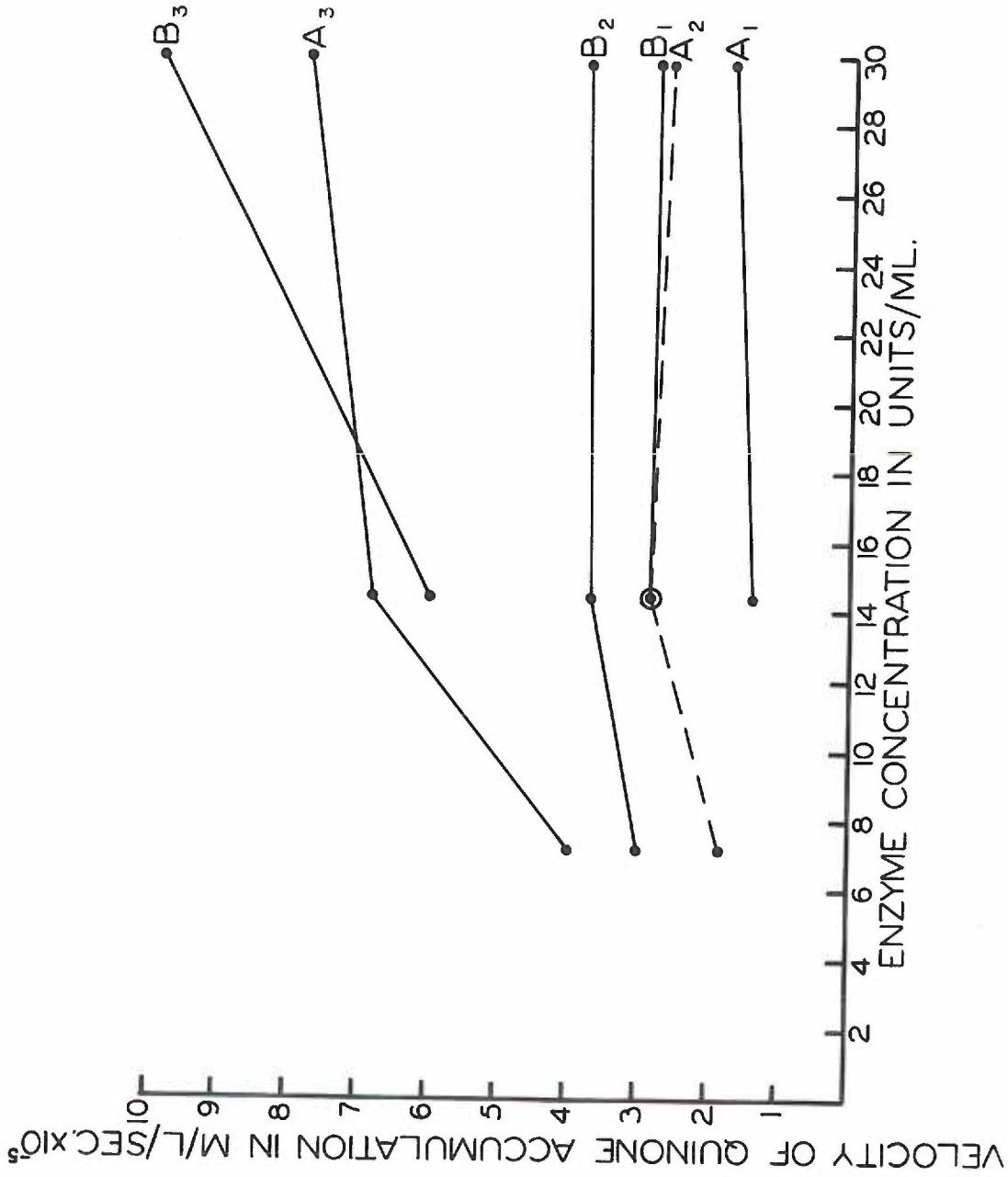

VELOCITY OF QUINONE ACCUMULATION IN M/L/SEC.x10⁵
1
2
3
4
5
6
7
8
9
10
ENZYME CONCENTRATION IN UNITS/ML.
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
B3
A3
B2
B1
A2
A1

Figure XXVI Comparison of quinone accumulation rate and maximum free radical concentrations at different oxygen concentrations.

A: .00835 M catechol

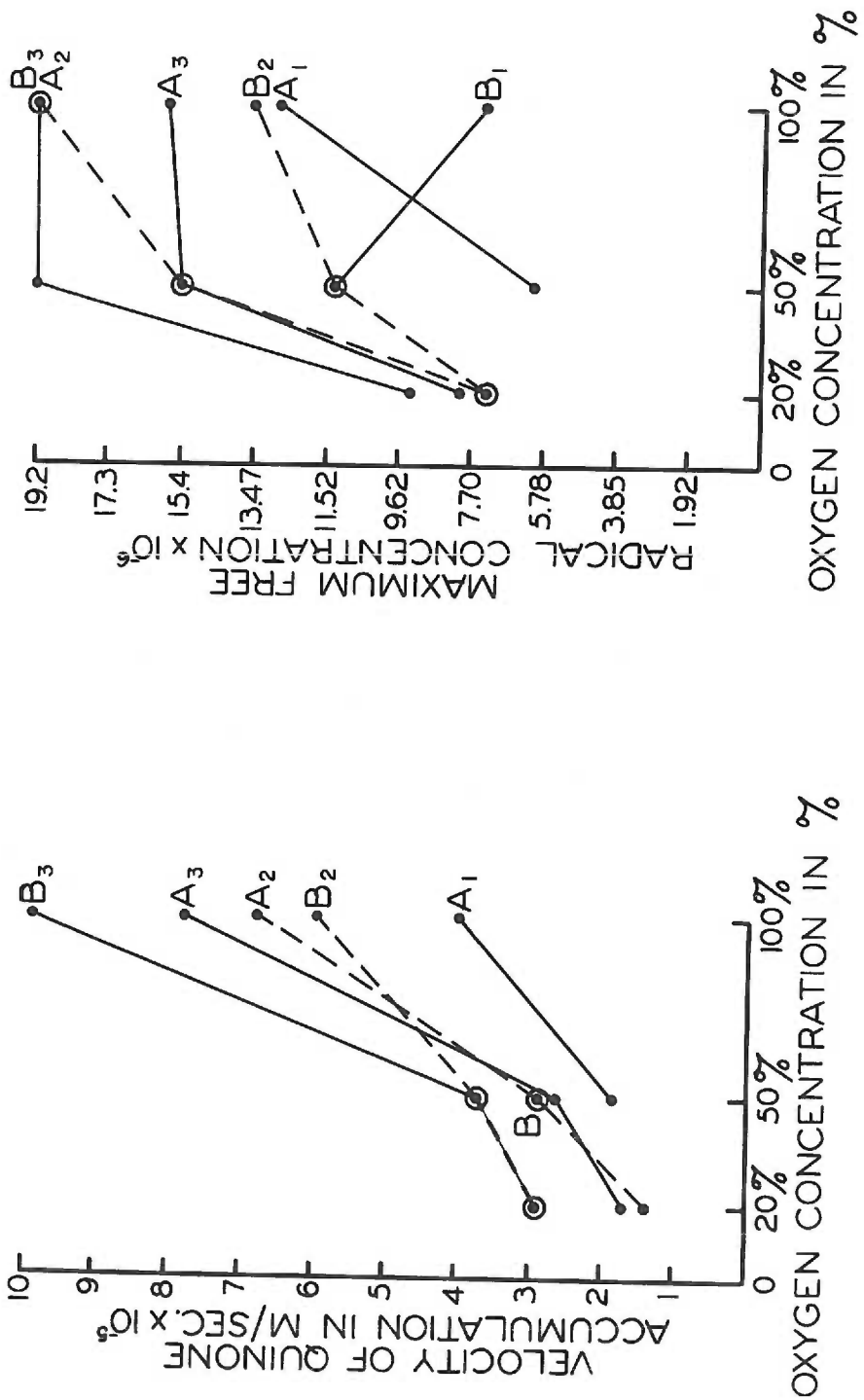
B: .0167 M catechol

1: 7.6 units/ml. tyrosinase

2: 14.4 units/ml. tyrosinase

3: 28.8 units/ml. tyrosinase

Figure XXVI


VELOCITY OF QUINONE ACCUMULATION IN M/SEC. x 10⁵
10
9
8
7
6
5
4
3
2
1
0
20%
50%
100%
OXYGEN CONCENTRATION IN %
B3
A3
A2
B2
A1
B0
MAXIMUM FREE RADICAL CONCENTRATION x 10⁵
19.2
17.3
15.4
13.47
11.52
9.62
7.70
5.78
3.85
1.92
B3 A2
A3
B2 A1
B1
0
20%
50%
100%
OXYGEN CONCENTRATION IN %

and catechol concentrations. Figure XXV shows the data for 20% oxygen saturation conditions. From these data the equilibrium constant is calculated. The calculations are summarized in Table I.

Repeated scans from 360-640 mμ show that the absorption at 390 mμ was mainly attributable to the amount of o-benzoquinone in the mixture. There was no appreciable shift in the wave-length of the peak as would be expected if some of the absorption were due to a new compound being formed of slightly different absorption characteristics. There was no great rise in absorption on either side of the peak to indicate a masking by an increase in general absorption from accumulating melenin. A straight line can be drawn through the maxima to confirm the straight line data of the previous work.

The catechol concentration at any time was estimated from the activity of the enzyme determined by the Miller-Dawson procedure. Each unit catalyzes the oxidation of 1.49×10^{-8}M catechol/ sec. when air is continually bubbled into the reaction mixture during the determination. In this procedure the catechol concentration is kept constant and no quinone accumulates in the system because of the presence of ascorbic acid. When 50% oxygen was used the rate of oxidation of a given enzyme preparation was 1.4 times what it was at 20% oxygen, and at 100% oxygen the ratio to that at 20% oxygen was 1.6. These data are collated in Table II.

Figure XXVII A comparison of quinone and free radical concentrations as a function of time. The time scale is expanded so as to demonstrate both variations on the same coordinates. The lines show the quinone accumulation and the curves show free radical accumulation and decay. A: 14.4 units/ml. enzyme, .00835 M catechol

B: 14.4 units/ml. enzyme, .0167 M catechol

C: 28.8 units/ml. enzyme, .00835 M catechol

D: 28.8 units/ml. enzyme, .0167 M catechol

All these data are for air-saturated conditions.

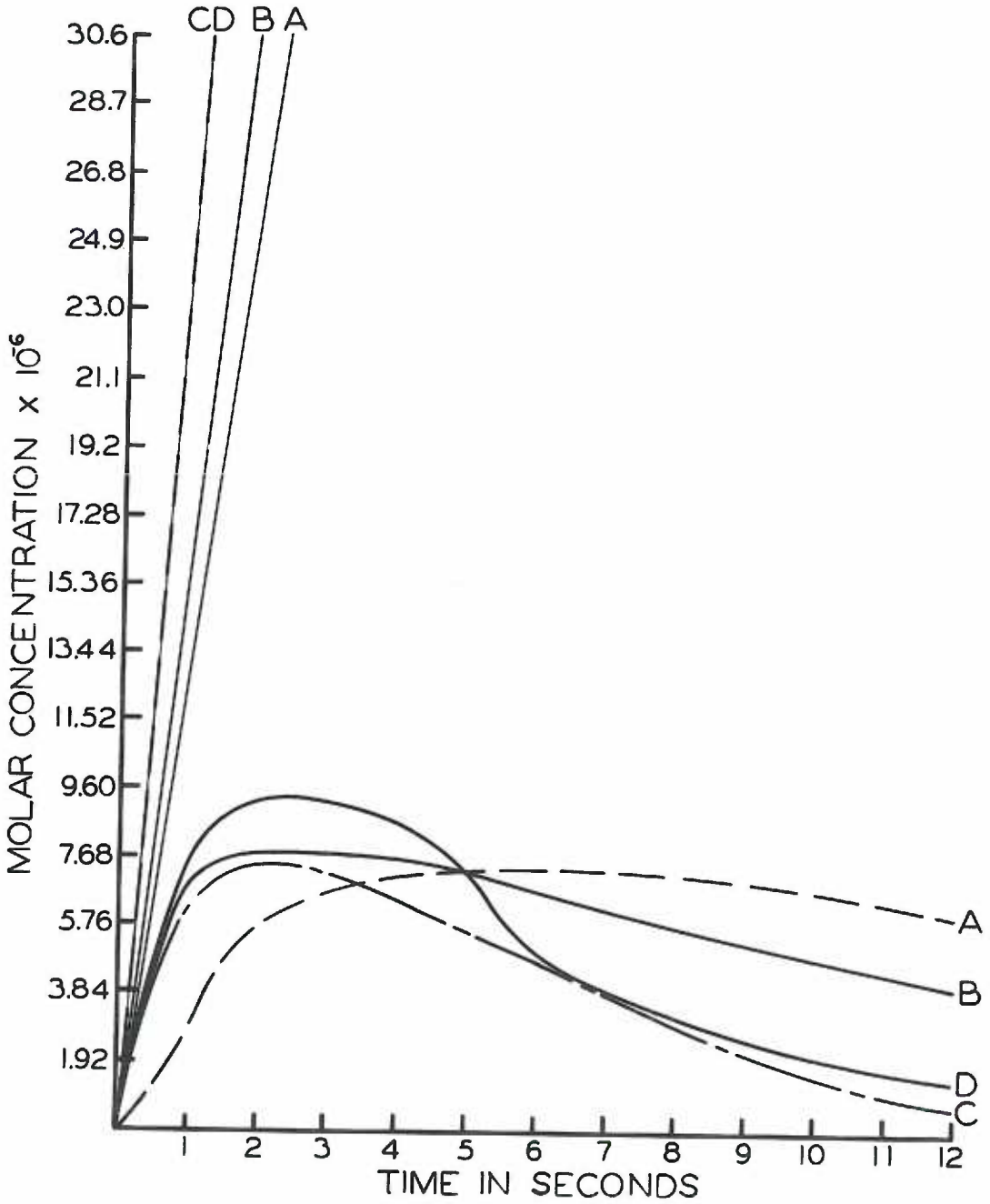

CD B A
30.6
28.7
26.8
24.9
23.0
21.1
19.2
17.28
15.36
13.44
11.52
9.60
7.68
5.76
3.84
1.92
MOLAR CONCENTRATION x 10^{6}
A
B
D
C
1 2 3 4 5 6 7 8 9 10 11 12
TIME IN SECONDS

TABLE I

SUMMARY OF DATA ON REACTION RATES & QUINONE FORMATION

#	O_2 pressure % atm.	initial Catechol conc. M	Enzyme units/ ml.	initial overall reaction rate M/sec. x 10^{-4}	Rate of Quinone Accumulation x 10^{-5}	Ratio dQ/dC	Ave.
1	20	.00835	14.4	2.14	1.38	.0644	
2		.0167		2.14	2.84	.1325	
3		.00835	28.8	4.28	1.70	.0397	
4		.0167		4.28	2.84	.0664	.076
5	50	.00835	7.2	1.5	1.85	.1235	
6		.0167		1.5	2.98	.1985	
7		.00835	14.4	3.0	2.84	.0994	
8		.0167		3.0	3.69	.1230	
9		.00835	28.8	6.0	2.70	.0450	
10		.0167		6.0	3.83	.0638	.109
11	100	.00835	7.2	1.71	3.98	.2320	
12		.00835	14.4	3.42	6.82	.1990	
13		.0167		3.42	5.96	.1740	
14		.00835	28.8	6.84	7.81	.1140	
15		.0167		6.84	9.94	.1450	.173

TABLE II

OVERALL REACTION RATE BY THE MILLER-DAWSON METHOD

#	% O_2	Time to oxidize 3 mg. ascorbic acid	$\frac{\text{time in air}}{\text{time in } O_2}$
1118	20	48 sec.	
			1.4
	50	32 sec.	
1123	20	44 sec.	
			1.6
	100	28 sec.	

DISCUSSION AND CALCULATIONS

Yamazaki (73), in his work with Mason and Piette, has worked out the kinetics of the oxidation of ascorbic acid by hydrogen peroxide in the presence of horseradish peroxidase. Ascorbic acid free radical was not stable under the conditions of their experiments, and its decay was described as a bimolecular process, either disproportionation, dimerization, or both. At steady state the rate of generation of the radical is equal to the rate of decay, and the steady state concentration of free radical can be expressed by the equation:

$$\left[AH\cdot\right]_s = \sqrt{\frac{E}{\left[\frac{k_d}{k_1\left[H_2O_2\right]}\right] + \left[\frac{k_d}{k_3\left[AH_2\right]}\right]}}$$

where AH_2 is the ascorbic acid concentration,

k_d is the decay constant of the free radical,

E is the total peroxidase concentration,

k_1 the velocity constant for the combination of peroxidase with H_2O_2 and

k_3 the velocity constant for the combination of compound II with ascorbic acid.

Since the hydrogen peroxide concentration is very much higher than the ascorbic acid concentration, it has little effect upon the free radical concentration, which can then be expressed by the equation:

$$\left[AH\cdot\right]_s = \sqrt{\frac{k_3 E \left[AH_2\right]}{k_d}}$$

This equation shows that the free radical concentration at steady state is proportional to the square root of the enzyme and substrate concentrations.

If tyrosinase should act by a free radical mechanism, and the catechol and oxygen concentrations were kept above the $2K_m$ values for both, the reaction rate would be independent of catechol concentration. If oxygen were also non-limiting throughout the reaction (according to Ingraham (29) it should be above 10% of an atmosphere at .01 M catechol if mushroom tyrosinase is like the prune enzyme) the reaction would be zero order with respect to oxygen, too. In such a case, the initial reaction velocity, which would remain constant for at least a short period, could be written $v = k_3 E$, where k_3 is the dissociation constant for a catechol-oxygen-tyrosinase complex (or a velocity constant for the reaction between a catechol-tyrosinase complex and oxygen) and E is the enzyme concentration. If k_d is a second order decay constant for o-benzosemiquinone, including disproportionation and dimerization, then the concentration of o-benzosemiquinone can be written, at steady state, as

$$\left[SQ\cdot\right]_s = \sqrt{\frac{v}{k_d}}$$

where v is the velocity of free radical formation. In this case, at the same overall reaction velocity, the

tyrosinase should give rise to the same type of free radical concentration vs. time curve as peroxidase at the same pH temperature. Actually, at pH 5.3, peroxidase showed the typical steady state behavior in the oxidation of catechol, while tyrosinase produced less than 1×10^{-7} M/sec. free radical at the same reaction velocity. From the above velocity it is seen that the reaction velocity is proportional to the square of the free radical concentration. Thus the ratio of reaction velocities of tyrosinase oxidation to peroxidatic oxidation being $2.6 \times 10^{-4} / 2.3 \times 10^{-4}$, the ratio of the squares of the free radical concentrations should be 1 . 2. It was actually $\frac{1 \times 10^{-14}}{16 \times 10^{-14}}$ or 0.0625 which is 5.2% of the expected value or less. This means that less than 6% of the product of tyrosinase activity can give rise to semiquinone directly.

At pH 7.6 both peroxidase and tyrosinase give rise to free radicals. At the same overall velocity the gross shape of the reaction curves is alike and the maximum free radical concentration is the same. However, a close look at the initial course of the reactions, as displayed in figure XXI , discloses a fundamental difference. The peroxidase curve shows that the initial production of semiquinone is so fast that it is not at first at equilibrium with the quinone and catechol in the system, but is at a

steady state condition, but is governed in free radical output by the equilibrium restriction from the beginning.

$$(\text{o-benzosemiquinone})^2 = K_E\ (\text{o-benzoquinone})(\text{catechol})$$

If o-benzoquinone were stable at this pH, and the only decomposition of the semiquinone were by disproportion, the free radical concentration as a function of time would obey the rule.

$$R = \sqrt{K_E(k_3Et)(C_o - k_3Et)}$$

The time course of the reaction would then be described by a parabola until oxygen or catechol were depleted below the $2K_m$ concentration, when the reaction velocity would decrease. In such a case the maximum free radical concentration would be reached at $\frac{C_o}{2K_3E} = t$. Its value would be $R_{max.} = \frac{\sqrt{K_E}\ C_O}{2}$, independent of enzyme concentration, dependent only on initial substrate concentration, and the expected curves for constant catechol concentration would be a series of parabolas with the same maxima but with different length of latus rectum. For double the catechol concentration, the height of the maxima should be doubled, but the latus rectum of each parabola should be the same as that for the same amount of enzyme at the lower catechol concentration. The relationship would cease to follow this law as soon as the catechol concentration fell low enough to

decrease the reaction velocity, but more than three quarters of the curve should have been traced before this occurred. In the flow experiments the oxygen concentration becomes rate-limiting so very soon that it is impossible to test the above relationship by the data obtained. In the recirculation experiments there is enough oxygen because it is bubbled constantly through the circulating reaction mixture and catechol, theoretically, to show the entire curve.

It is easily seen from the curves obtained by the recirculation experiments (Figs. XXIV and XXIII) that the reaction products are not stable, even for the first few seconds of the experiment. It is, of course, obvious from the accumulation of melanin in the reaction mixtures that further reaction, either oxidation or polymerization or both have taken place. The above theoretical equation should then be corrected to allow for decomposition of the quinone or free radical or both.

Neither the quinone nor the free radical are stable in alkaline solution. Side reactions, that is, besides the dismutation equilibrium, of all possible orders can take place. The bimolecular processes seem most probable as first steps in polymerization, more probable in dilute solution than reactions of higher or lower order. Making this assumption, and if, as seems true with tyrosinase, o-quinone is the first product of

reaction, its decomposition is most important because its total effect is largest

$$\frac{dQ}{dt} = k_3E - k_dQ^2$$

where k_d is the bimolecular constant for the quinone

$$t = \frac{1}{k_d}\int \frac{dQ}{\frac{k_3E}{k_d} - Q^2} = \frac{1}{2\sqrt{k_dk_3E}} \ln \frac{\sqrt{\frac{k_3E}{k_d}} + Q}{\sqrt{\frac{k_3E}{k_d}} - Q}$$

$$Q = \sqrt{\frac{k_3E}{k_d}} \frac{(e^{2t\sqrt{k_dk_3E}} - 1)}{(e^{2t\sqrt{k_dk_3E}} + 1)}$$

$$Q_{max.} = \sqrt{\frac{k_3E}{k_d}}$$

Then, if equilibrium obtains

$$\frac{R^2}{\left[\sqrt{\frac{k_3E}{k_d}} \frac{(e^{2t\sqrt{k_dk_3E}} - 1)}{(e^{2t\sqrt{k_dk_3E}} - 1)}\right]\left[C_o - k_3Et\right]} = K_E$$

Simplifying, $R = \sqrt{K_E} \sqrt[4]{\frac{k_3E}{k_d}} \sqrt{\frac{(e^{2t\sqrt{k_dk_3E}} - 1)(C_o - k_3Et)}{(e^{2t\sqrt{k_dk_3E}} + 1)}}$

$$t = 0, \quad R = 0$$

$$R \sim \sqrt{K_E} \ , \ \sqrt[4]{\frac{k_3 E}{k_d}} \ , \ C_o$$

At end of reaction, $k_3 E t = C_o$, $R = 0$

Since $$\sqrt{\frac{(e^{2t\sqrt{k_d k_3 E}} - 1)}{(e^{2t\sqrt{k_d k_3 E}} + 1)}} < 1$$

$$R_{max.} < \sqrt{K_E} \cdot \frac{C_o}{2}$$

Simplification by Maclaurin's series expansion (not too accurate an approximation)

$$e^{2t\sqrt{k_3 k_d E}} = 1 + 2t\sqrt{k_d k_3 E} + \text{terms of higher order}$$

$$R = \sqrt{\frac{K_E k_3 E t (C_o - k_3 E t)}{1 + t\sqrt{k_d k_3 E}}}$$

This equation, then, gives the free radical concentration formed by reverse dismutation from o-quinone and catechol during tyrosinase-catalyzed oxidation of catechol, assuming bimolecular decay of o-quinone and o-semiquinone. Then the free radical concentration is dependent upon the $\sqrt{K_E}$, the initial catechol concen-

tration, the enzyme concentration and the time.

This equation, however, is inconsistent with the linear accumulation of quinone found by spectrophotometric measurements. It is also true that the accumulation of quinone is from 4% to 20% as fast as the disappearance of catechol. If the disappearance of catechol is assumed to be linear until the catechol concentration falls below $2K_m$, and if the accumulation of quinone is linear, as appears to be the case, it follows that the decay of quinone may be zero order with respect to quinone concentration. If the assumptions are correct, this indicates that either the formation of melanin does not depend upon the size of the quinone pool in the reaction mixture or that the spectrophotometer is not measuring quinone only. This fact can be expressed mathematically as follows:

$$\frac{dQ}{dt} = k_d \cdot k_3 E = k_3 E - (1 - k_d) k_3 E$$

k_d is .04 to 0.20

It is possible that the semiquinone reacts with oxygen, competing with the enzymatic oxidation for the oxygen in solution.

CALCULATION OF FREE RADICAL CONCENTRATION

In electron spin resonance spectroscopy, it has been found that the integrated intensity of the absorption curve is proportional to the free radical concentration. The theoretical expression for the relation between the power absorbed and the number of spins is given by the equation which has been derived on page 22:

$$N_0 = 1.02 \times 10^{31} \frac{\text{Power absorbed}}{H_1^2 \omega \omega_0 g(\omega - \omega_0)} \cdot T$$

In actual practice, however, it has been found to be more satisfactory to use a standard in which the number of spins can be estimated by some other means and a proportionality factor worked out that takes account of the peculiarities of the equipment, such as the sensitivity of the recorder, certain characteristics of the crystal and the gain of the amplifier system, etc. Various standards have been employed, such as charred sugar, ground coal, irradiated salt crystals, crystals in which a small number of paramagnetic ions are dispersed in the lattice, diphenylpicrylhydrazyl, and solutions of free radicals which can be titrated for their free radical content. We used peroxylamine disulfonate, prepared and standardized titrimetrically by Dr. Nerni. He and Dr. Yamazaki placed various concentrations of this material in the cell used for

Figure XXVIII Area moment of the o-benzosemiquinone signal plotted against the height of the derivative peak chosen as a measure of free radical concentration. The data taken from the four identification curves mentioned in the text, one of which is illustrated in Figure XI.

Figure XXVIII

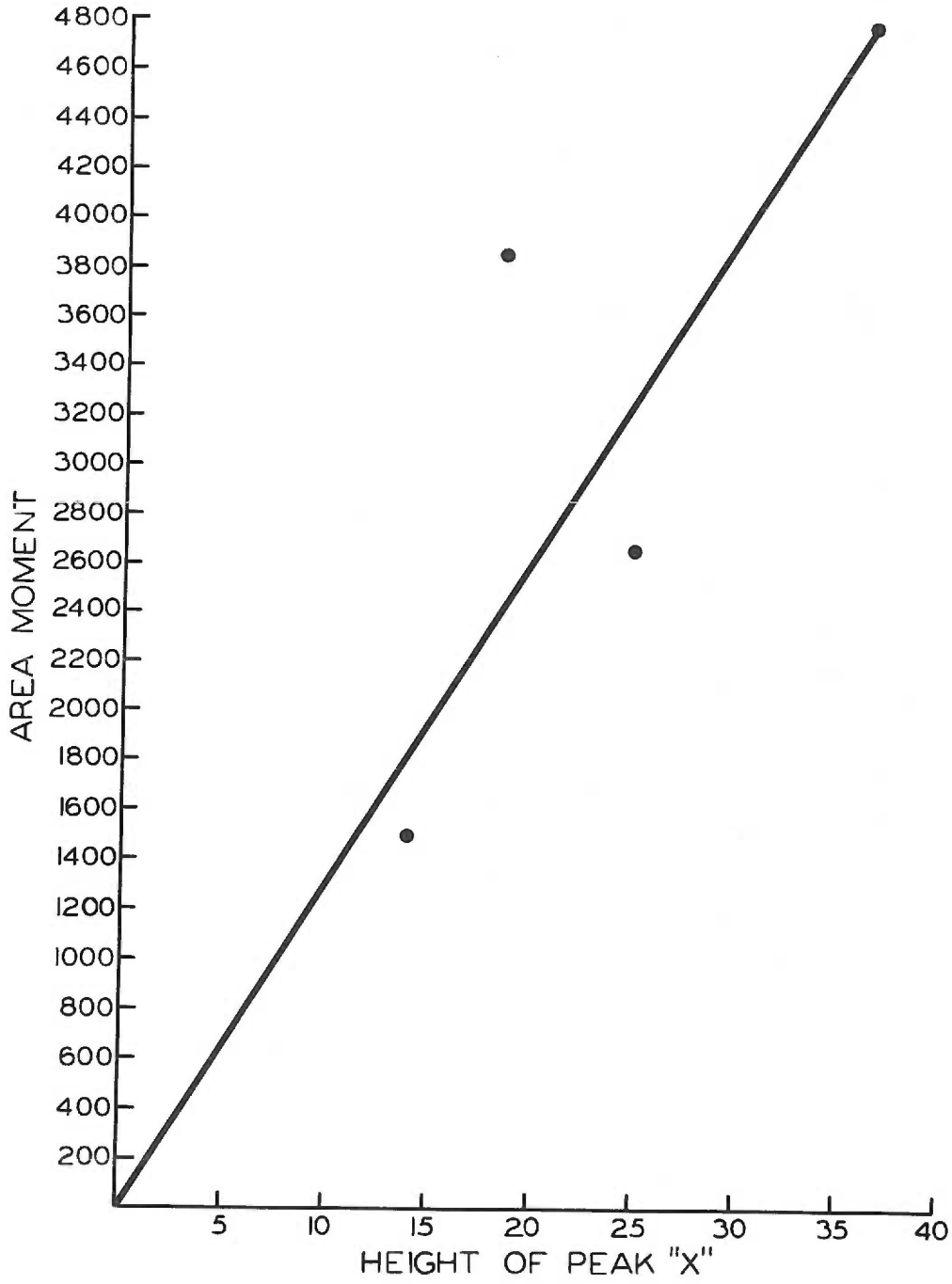


TABLE III

EQUILIBRIUM CONSTANT CALCULATIONS

# from Table I	Time, sec.	Q x 10^5	C x 10^3	R x 10^6	K_E x 10^4
1	1	1.38	8.14	2.70	.647
	3	4.14	7.71	6.74	1.42
2	1	2.84	16.5	6.35	.859
	3	8.52	16.1	7.30	.388
3	1	1.70	7.92	7.12	3.76
	3	5.16	7.06	7.89	1.72
4	1	2.84	16.3	7.7	1.28
	3	8.52	15.4	9.24	.649
5	1	1.85	8.2	3.08	.625
	3	5.55	7.9	5.77	.760
6	1	2.98	16.5	3.46	.244
	3	8.94	16.3	6.82	.32
7	1	2.84	8.05	5.78	1.46
	3	8.52	7.45	12.9	2.62
8	1	3.69	16.4	1.92	.06
	3	11.1	15.8	4.81	.13
9	1	2.70	7.92	8.66	3.41
	3	8.10	6.55	15.2	4.36
10	1	3.83	16.3	15.4	3.79
	3	11.5	14.9	19.6	2.25
11	1	3.98	8.18	2.89	.22
12	1	6.82	8.01	6.74	.83
13	1	7.81	7.67	6.35	.67
14	1	5.96	16.4	6.74	.46
	3	17.9	15.7	11.2	.44
15	1	9.94	16.0	7.70	.37
	3	29.8	14.7	18.9	.81

K_E average for 20% oxygen = 1.34×10^{-4}

K_E average for 50% oxygen = 1.99×10^{-4}

Q = quinone concentration

C = catechol concentration

R = o-semiquinone concentration

pH = 7.6

buffer = 0.1 phosphate

the experiments described in this paper and recorded the derivative trace of the absorption curve at the same settings of modulation amplitude, time constant, and scan speed used in the experiments.

The area under the absorption curve was calculated by the use of the area moment equation:

$$\int_{-\infty}^{\infty} x\, f'(x)dx = \int_{-\infty}^{\infty} f(x)dx$$

Three different derivative curves were integrated by the above formula giving 133 x 10^4, 138 x 10^4 and 139 x 10^4 squares of coordinate paper/unit gain/mole/liter, respectively, with an average of 137 x 10^4. At 500 gain this number becomes 685 x 10^6 sq./M (M = mols./liter). This is, then $\frac{1}{685}$ x 10^{-6}M/sq. of area. The factor is multiplied by 130 on page 136 to obtain the new factor 1.89 x 10^{-7} by which the pen deflection in the recirculation experiments was multiplied to obtain free radical concentration.

Four different derivative tracings of the absorption curve of o-benzosemiquinone were obtained from identification runs from the enzyme experiments. The three well separated lines of each derivative curve were integrated separately by the method of area moments, and the resulting three areas added to give

the area under the total absorption curve. This procedure corrects in part for variation in base line occurring when such a broad signal is traced. The areas obtained from the four separate signals were plotted against the heights of the derivative peaks selected for the index of free radical concentration in the experiments. A straight line drawn through the points (Fig. XXVIII) showed reasonable regularity in this relationship, and the slope, 130, was taken as the conversion factor from peak height to area. The factor, 1.89×10^{-7}, is used to convert the pen deflection in the experiments to free radical concentration. See page 94.

CALCULATION OF QUINONE CONCENTRATION

From the relation (66) $C/D = 4.42$, where i, the length of the cell, is 1 cm., the proportion $C_1 l_1/D_1 = C_2 l_2/D_2 = 4.42$ gives $l_2 = (4.42)(0.385)/44.2 = .0385$. The extinction coefficient for o-benzoquinone in water (47) is log = 3.262. The o-benzoquinone concentration in the solution is then $D_{390}/.0385(1825)$ M. $= 1.42 \times 10^{-2} D$ (See Table I).

CALCULATION OF CATECHOL CONCENTRATION

Since the consumption of catechol is given by the expression $k_3 Et$ and is proportional under the conditions of the experiment to the enzyme concentration only, the enzyme concentration in units/ml. is multiplied by

1.49×10^{-5} to get mols. catechol per liter oxidized. At 50% oxygen this number is multiplied by 1.4 and at 100% oxygen by 1.6 as calculated from the data of Table II.

THE EQUILIBRIUM CONSTANT

The equilibrium constant is determined by the relation

$$K_E = R^2/QC$$

The data so calculated are summarized in Table III.

The systematic errors inherent in the determination of the concentration of free radicals in a sample by electron spin resonance are:

1. Pen drag as the absorption curve is scanned, due to friction
2. Hysteresis of the magnet. The slower the scan, the less the effect of this error
3. Too long or too short a response time in the pen circuit. Too short a response time magnifies the noise because of the increased sensitivity but too long a response time tends to "iron out" or flatten the peaks in the hyperfine structure
4. Errors in integration. If the pen tracing were perfect, with no noise spikes, this error could be the most important one, as slight errors in measurement of the squares at any point on the

curve are greatly magnified in integration, especially on the edges of the curve. Integration error could easily be minimized by use of a planimeter, or by the standard methods of graphic integration, such as by weighing sections of carefully drawn curve on paper of uniform thickness. It is possible to magnify such curves with any desired degree of accuracy. None of these methods avail in increasing accuracy unless the curve is accurately drawn in the first place.

5. Drifting base line. This is one of the worst errors in a broad absorption curve as it causes the largest deviations on the edges of the curve where they count the most because they have the largest "x" or field component in their moments.
6. Modulation amplitude. Too great a modulation amplitude causes "modulation broadening" smearing out hyperfine structure. About half the line width in gauss is recommended. For the workdescribed in this paper the setting 25 on the machine was used.
7. Chart speed. The time scale must be spread out enough to show the full shape of the curve.

The resultant of the above factors is an error of 20% in the estimation of free radical concentration upon our machine, using double integration, according to

the method of moments, of the derivative curves. Duplicate runs by Dr. Narni on standard samples with our spectrometer show a deviation of 20% in spin concentration calculations which is also the experience of Dr. Piette on his machine in Palo Alto, California. This error is compounded when the machine is set on a single "peak" to follow the reaction curve. It is hard to be sure the machine has remained in perfect adjustment throughout the experiment, as the adjustment cannot be checked during the run. The spectrometer must be set on the same "peak" each time as the variation of the heights of the different peaks with curve area variation is different.

It is hard to know the exact meaning of the "peak" height recorded, for the height of the peak during scanning may well be less than the height when the machine is held directly on the peak value. Hysteresis in the magnet, pen time response and mechanical lag are all important in determining peak height during scanning, but do not affect peak height at constant free radical concentration when the magnetic field is held constant. Pen time response is important when the free radical concentration is changing and must be fast enough to keep up with the rate of that change.

In order that the flow data should be reproducible the rate of flow during the run must be measured.

This was done by a coarse regulation of pressure in the flow apparatus and a measurement of the volume of liquid discharged in a given time for each run. The volume of the cell from the mixing chamber to halfway up the flat part was measured and the time after mixing calculatedffrom the relation:

$$\frac{\text{vol. of cell} \times \text{time}}{\text{ml. flow}} = \text{time after mixing when reaction mixture reaches center of cell}$$

In fact, the calculation of equilibrium constant which is not very constant shows that the system may not be at equilibrium, although oxygen concentration is kept relatively constant by continuous bubbling and catechol concentration is well above the Km given for catechol, during the first 30 seconds of the reaction. Frieden reports the Km for catechol as 1×10^{-5}. At a constant utilization rate of 2×10^{-4}M/sec. the .01M catechol would be half gone, or at 5×10^{-3}M at 20 seconds and down to 2×10^{-5} only after 45 seconds or more. If catechol is reformed by the reaction of quinone with reduced polymer its concentration would decrease more slowly. The net effect upon the equilibrium constant would be to make it seem larger than it actually is, but the relative variation in catechol concentration brought about by such an effect is small as long as the catechol is above 5×10^{-3}M. Variation in quinone concentration has much more effect upon the equilibrium

constant because the total quinone concentration is small.

Ingraham (29) points out that the Km values for substrates of enzymes with two substrates are independent, and that in the case of prune polyphenol oxidase the Km of oxygen varies with the catechol concentration up to about 25 mM in catechol. His Km for the catechol concentration was about 5% of an atmosphere. He restricted the further oxidation of the quinone first formed and any extraneous catalytic action of the oxidase upon the reaction products, by using ascorbic acid to keep the quinone reduced and the catechol constant. His pH was 5.3, so that he did not have appreciable radical in the system either. He concluded that the enzyme combines with oxygen first because the limiting Km at low concentration for O_2 is the same for all substrates regardless of structure.

From the graph of Figure XXV it is seen that increasing enzyme concentration does not increase quinone accumulation, catechol remaining constant at 50% oxygen or less. Increasing catechol concentration seems to exert an inhibiting effect at lower enzyme concentration and high oxygen. Possibly this is further indication that the enzyme combines with oxygen first, and that further oxidation of reaction products competes with the enzyme for the oxygen in the system. When the enzyme present is fully oxygenated there seems

to be some inhibition by high catechol concentrations. (Fig. XXVI)

In the recirculation experiments of Figs. XXIII and XXIV the initial rise and maximum value of free radical concentration described by the curves may be low because the response time of the recorder pen may not be short enough to keep up with the changes taking place in the cavity of the instrument. It is possible that the true peak of free radical concentration comes within the first second of the reaction, in which case the pen could not respond fully. There is also a time lapse between the addition of enzyme to the solution and complete mixing, plus the 0.2 seconds it takes for the solution to reach the center of the cell. If the measured free radical concentration is lower than the actual concentration, the equilibrium constant will be low. In the 100% oxygen experiments the pen deflection is much greater than in the 20% oxygen runs. The peak also comes earlier. The much lower equilibrium constant calculated from the 100% oxygen data indicates that the above factors are operative.

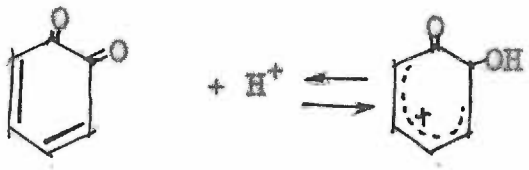
The early decay of the free radical indicates that it does not remain at equilibrium with the quinone and catechol in the system, but that it decays independently by other mechanisms than disproportionation. The equilibrium constant from the 20% oxygen data tends

towards a fairly steady value for the first three seconds of reaction, indicating that the free radical is nearly at equilibrium with quinone and catechol. Calculations based on data taken later in the reaction give erratic results, showing that the equilibrium is being upset by subsequent reactions, either of quinone or semiquinone. Since the quinone has been shown only to increase at a steady rate for a period of two minutes or more (Fig. XXVII), it is doubtful if the equilibrium is upset by that factor in the reaction. The catechol may not be decreasing steadily at the theoretical overall rate because in this system it can be reformed from quinone by reaction of quinone with reduced polymer to give catechol and oxidized polymer. Because the catechol concentration is high relative to that of the other components of the mixture, small changes due to the oxidation effect should not have much effect upon the equilibrium constant. Free radical decay is not explained by decrease in quinone or catechol. concentrations, since quinone increases and the decrease in catechol concentration is not sufficient to account for it. If the free radical were formed from quinone and catechol to begin with and not directly by enzyme action, it is highly improbable that the free radical concentration could have exceeded its equilibrium value at any time, so that its disappearance is not a matter of dismutation.

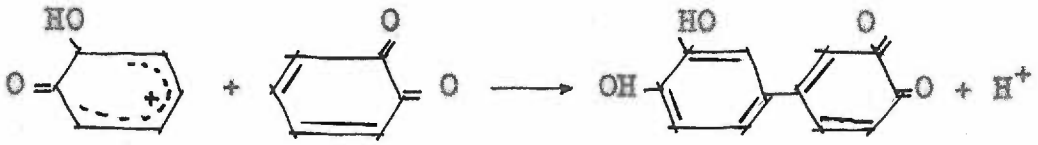
Forsyth and Quesnel (19) have studied the oxidation products of catechol in the presence of tyrosinase as well as in the presence of certain inorganic oxidizing agents such as silver nitrate and iodates. They found three isomeric tetrahydroxy-diphenols and diphenylenedioxide 2, 3-quinone among the reaction products when the catechol concentration was 5×10^{-3}M or greater, identifying them by paper chromatography. They used McIlvaine's buffer at pH 5.1, bubbling air slowly through the solution, during the reaction. These products point to the possibility of a free radical mechanism of some type, but not necessarily a semiquinone intermediate, although a semiquinone intermediate is not logically impossible. It is deduced from the hyperfine structure in the electron spin resonance spectrum of o-benzosemiquinone (54) that the free electron is not entirely limited to the vicinity of the oxygen atoms of the radical indicated by the structural formula, but spends some time near the hydrogen atoms all around the benzene ring with which it is in resonance. This indicates that it is in communication with the resonating pi-orbital system and there is a finite probability that it would be localized at a given instant in time on any one of the carbon atoms of the ring. It is seen from the structure of this particular resonance that two of the carbon atoms are favored over the other two

in probability. Michaelis (55) has clearly demonstrated many examples of semiquinone production by inorganic oxidation and it is safe to state that the inorganic oxidation can produce the above diphenyl compounds by the free radical mechanism. The free radical may abstract hydrogen from another molecule, being reduced to catechol and creating a new free radical with the electron more highly localized on the ring for an instant. Two of these new radicals could then dimerize to the diphenyl compound. Or, two semiquinone radicals with temporarily high electron densities on ring carbons could dimerize, expelling the protons present on the ring, which would then be picked up by the electron pair resonating to the oxygen. Instead of dimerizing oxygen to oxygen as one might expect for some types of free radicals, dimeric semiquinones with para positions unsubstituted can rearrange to the more stable diphenylenedioxide configuration.

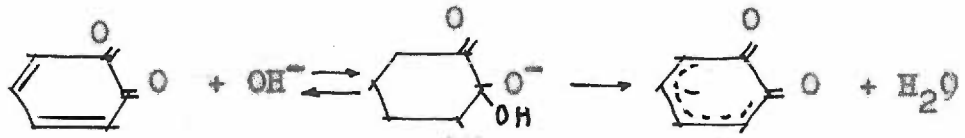
The products found by Forsyth and Quesnel (19) could also have been formed from o-benzoquinone by non-free radical polymerization catalyzed by acid or base. In the presence of hydrogen ions the resonating quinone could react as follows:



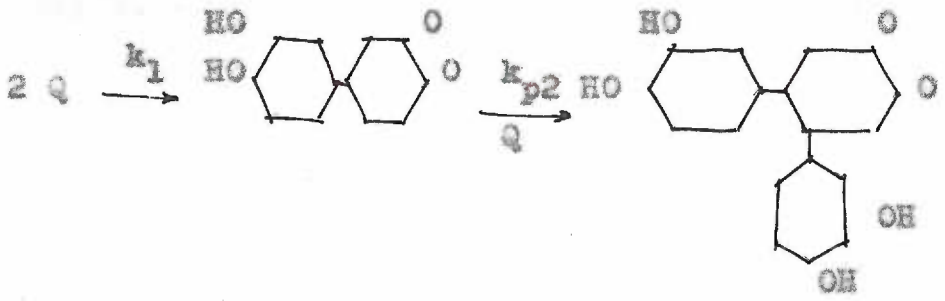
The positive charge on the ring would be distributed among the four resonating positions because they are all either ortho or para to an oxygen atom. Then the condensation would take place:



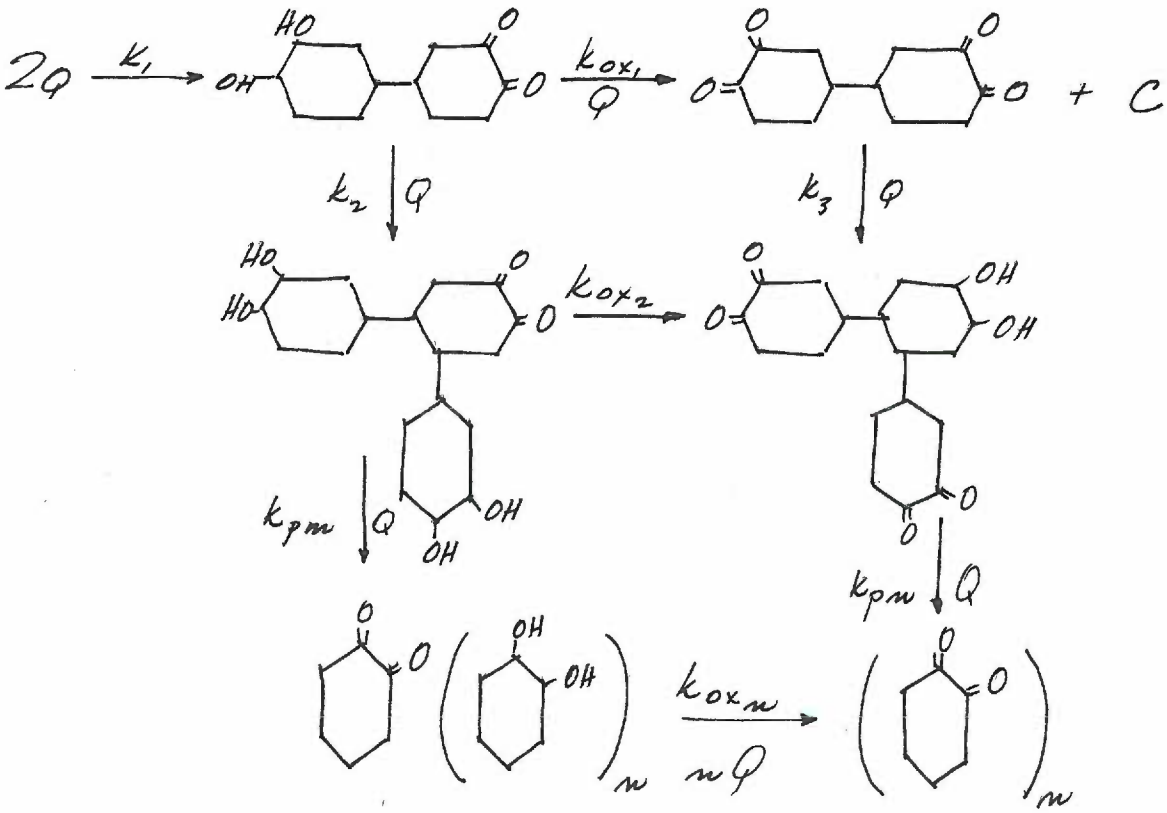
All possible isomers are logical results of this mechanism. The mechanism of base catalysis is similar:



If acid or base catalysis were dominant in accounting for the disappearance of quinone from an enzyme system, the manner of formation of melanin could be envisioned as a non-enzymatic polymerization of quinone, starting with the products of Forsyth and Quesnel, as follows:



The diphenyl compound could then condense with another molecule of quinone to form a trimer, continuing in the same way to form the higher polymers, or it could react with a molecule of quinone to form catechol and a fully oxidized quinonoid dimer. Similar oxidations could take place at any stage of the polymerization and mixed types of polymers would no doubt exist in the melanin, according to the following equations:



If the assumption is made that $k_{p2} = k_{p3} \text{ ---- } k_{pm} = k_{pn}$ (as is done in the treatment of free radical chain reactions by Walling (69)) and that $k_{ox_1} = k_{ox_2} \text{ ---- } k_{ox_n}$ where k_{p2}, k_{p3}, etc. are polymerization constants at the various stages of the reaction and k_{ox_1}, k_{ox_2}, etc. are the rate constants for oxidation by quinone at the various stages as indicated above, k_p may be used to designate the polymerization constant and k_{ox} the oxidation constant for the system. Then the overall rate of disappearance of quinone would be:

$$v = k_1 Q^2 + k_{ox} Q \left[\text{reduced polymer}\right] + k_p Q \left[\text{polymer}\right]$$

The rate at which quinone would accumulate in the system when generated by the enzyme at the constant rate $k_3 E$ is:

$$\frac{dQ}{dt} = k_3 E - k_1 Q^2 - k_{ox} Q \left[\text{reduced polymer}\right] - k_p Q \left[\text{polymer}\right]$$

If polymerization stopped spontaneously when the molecules reached a certain size and k_t is the chain termination rate constant, the change in polymer concentration can be written:

$$\frac{d\left[\text{polymer}\right]}{dt} = k_1 Q^2 - k_t \left[\text{polymer}\right]$$

At steady state

$$k_1 Q^2 = k_t [\text{polymer}]$$

$$[\text{polymer}] = \frac{k_1 Q^2}{k_t}$$

$$\frac{d[\text{reduced polymer}]}{dt} = k_1 Q^2 + k_p Q [\text{polymer}] - k_{ox} Q [\text{reduced polymer}]$$

At steady state

$$k_{ox} Q [\text{reduced polymer}] = k_1 Q^2 + k_p Q [\text{polymer}]$$

Then

$$\frac{dQ}{dt} = k_3 F - 2 k_1 Q^2 - \frac{2 k_p k_1 Q^3}{k_t}$$

The above equation does not integrate to a linear relationship between quinone and time.

Two general types of mechanism may explain our observations: one in which the enzyme is assumed to catalyze polymerization, and the other according to which the activity of the enzyme is restricted to o-quinone formation the other characteristics of the system arising from the relationships between catechol, o-quinone and their non-enzymic reactions.

According to the first mechanism, if the same

products are formed by enzymatic oxidation as are formed by an inorganic free radical oxidation mechanism, it is only logical to suppose that the enzyme acts by free radical mechanism.

The results of our work show that at low pH not more than 6% of the oxidation could go by this route. It is possible, however, to conceive an enzymatic mechanism which would produce the substances found without the presence of semiquinone in the system. If the enzyme combines with oxygen first, and the attack upon the substrate takes place through the oxygen by removal of a hydrogen atom to the body of the enzyme, and if two of these molecules were being attacked at the same time at spots close together on the enzyme, it seems likely that the unpaired electrons on the substrate residues would pair at the enzyme surface, producing the dimer immediately. If the substrate molecule were held at two places on the surface of the enzyme, the second place could be released simultaneously with dimerization. The dihydroxy diphenyls are substrates for the enzyme also and further oxidation would take place readily. (19)

The appearance of quinone in the system at favorable pH is accounted for also in the above explanation. When the quinone is stable, the intramolecular resonance permits some of it to break away from the enzyme without

dimerization. The probability of this event is increased in proportion to the stability of the quinone and semiquinone. The semiquinone is produced from the quinone. Under reducing conditions, as in the presence of ascorbic acid, quinone could be removed from the enzyme by reaction with the ascorbic acid, but under oxidising conditions, as at pH 7.6 in the presence of excess oxygen, mostly polymer would appear in the solution. This hypothesis would also account for the lower amount of overall activity and lower rate of quinone accumulation at lower oxygen pressures. There are actually two reactions competing for the oxygen on the enzyme as well as free radical scavenging the oxygen in the solution.

It must be assumed in such a case that the cuprous ion is bound to the enzyme protein by a conjugated reducing system with a well-developed pi-orbital system similar to the oxyhemoglobin complex. This idea is compatible with the fact that copper can be removed from tyrosinase by dialysis against cyanide and that the enzyme is inactivated by copper chelating agents. It is also compatible with the observation of Kubowitz (39) that the enzyme combines with CO only as it is reduced by catechol. Although the specificity of tyrosinase is not at all narrow, including many dihydric phenols and encompassing two altogether different types of

activity, certain limitations of its activity point to a steric pattern which might place two of the coppers per molecule relatively close together, possibly in a crevice in the molecule which could contain ancillary groups for completing electron transfer to the substrate.

If the enzyme combines with oxygen first, the reaction rate depends upon the oxygen to enzyme ratio until the enzyme is saturated with oxygen, i.e. until oxygen is at equilibrium with the total number of oxygen-binding sites on the enzyme molecule. In the case of tyrosinase that number is unknown. Krueger (32) recently reported several tyrosinases separable by column chromatography containing different copper contents. If, however, there are 4 Cu per enzyme molecule (upon which several other investigators have agreed) and each Cu can bind an oxygen molecule, it is to be expected that 4 oxygen molecules could be bound to one enzyme molecule. It is likely, however, that not all the sites would be occupied at equilibrium. Whether the number of sites occupied is affected by the enzyme concentration or physical state is a matter of conjecture.

Assuming the above premises correct, at low oxygen pressures (air) and low enzyme concentrations the velocity of the enzyme reaction could be proportional to the enzyme concentration. It is. The Miller-Dawson assay technique is founded upon this principle. The

reaction velocity does increase with increased oxygen pressure and the effect is less at low enzyme concentrations than at high ones, as expected.

The K_m for catechol is a measure of the enzyme's capacity for holding catechol and obviously depends upon the fraction of the enzyme that is activated by binding oxygen. The apparent enzyme concentration is not the true value. It is also true that at high catechol concentration, after the reaction has been going long enough to build up some of the products found by Forsyth and Quesnel (19) these products can compete with the catechol, inhibiting the reaction and using up oxygen.

$$E + ?O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E(O_2)_?$$

$$E(O_2)_? + AH_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E(O_2)_{?-1}\,(O) + H_2O + A$$

or

$$E(O_2)_? + 2\,AH_2 \underset{k'_4}{\overset{k'_3}{\rightleftharpoons}} E(O_2)_{?-1}\,(O) + H_2O + (AH)_2$$

$$(AH)_2 + A \longrightarrow AH_2 + A_2$$

where AH_2 signifies catechol.

According to the second general mechanism, in which enzyme plays only the role of generating o-quinone from catechol, the observed rates of free radical formation and decay and o-quinone accumulation can be explained only by acknowledging several uncertainties in the determination of enzyme activity and catechol and o-quinone concentrations during the procedures we have employed.

Dawson (62) stated that tyrosinase is very sensitive to surface inactivation. The violent bubbling necessary to keep the oxygen concentration as high and constant as possible during the reaction would lead to an early dropping off of catechol oxidation rate due to enzyme inactivation.

The regeneration of catechol in the polymerizing system would alter the catechol concentration. Catechol concentration could not be measured in these experiments. The ultraviolet absorption peak of catechol is soon masked by the general melanin absorption when the reaction begins. Another method of estimating catechol concentration must be devised. If the enzyme could be "killed" at timed intervals during the reaction and the catechol quantitatively extracted from the reaction mixture, it could be measured. Even so, the overall catechol concentration does not change very much in the first two minutes of the reaction. The above effect would only tend to prolong the period in which the

velocity of the reaction is constant by slowing down the disappearance of catechol.

Reaction inactivation caused by bubbling should have a random effect upon the overall reaction rate, and indirectly upon the quinone accumulation rate. The quinone accumulation rate is very steady for the first two minutes and quite reproducible.

Although the spectrophotometer was started before the beginning of the reaction each time, its pen has a time constant of 1 second. There was a mixing time and 0.2 second for the solution to travel to the center of the cell. The course of the reaction is uncertain for the first second at least. If the quinone accumulation were not linear during this period the overall quinone accumulation might not be. In this case, a different interpretation of the results of these experiments would be necessary. Further work with rapid flow techniques is indicated.

SUMMARY AND CONCLUSIONS

1. Tyrosinase is a "two-electron" transfer enzyme. The first product of the oxidation of catechol catalyzed by tyrosinase is o-benzoquinone. Any semiquinone which occurs in the system at favorable pH is formed by the reaction of the quinone with catechol.

2. The o-benzoquinone formed by enzymatic oxidation is fairly stable at pH 7.6 and accumulates linearly in the system. The concentration of free radical at any time does not indicate that it is at equilibrium with the quinone present. The equilibrium constant is at least 1.5×10^{-4}.

3. There is some evidence to confirm Ingraham's statement that the oxygen combines with the enzyme first. It also confirms his observation that the K_m for catechol depends upon the oxygen concentration, and the K_m for oxygen upon the catechol concentration.

4. Although o-benzoquinone is released by the enzyme into the reaction mixture rather than o-benzosemiquinone, it is not the main product of enzyme action under the conditions of these experiments. Probably some polymerization takes place on the enzyme surface, coincident with quinone formation. The polymers would be quinonoid and oxidize ascorbic acid.

These conclusions are supported by data from the electron spin resonance spectrometer, which measures

free radical concentration directly, in flow and recirculation experiments, from the Cary recording spectrophotometer which measures quinone concentration by its absorption at 390 mμ , and from other ancillary data in traditional experiments. Tyrosinase has been prepared from mushrooms by a method improved in our laboratory and adapted to our equipment for large-scale production.

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