Studies of the action of molecular singlet oxygen on proteins and amino acids
by James Ross Fischer

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University

Abstract:
The effects of chemically and/or microwave discharge generated singlet oxygen on proteins and their constituents were shown to parallel the effects of dye sensitized photooxidation. This supports the contention that dye sensitized photooxidation is mediated by singlet oxygen. Chromatographic comparison of the products of oxidation by microwave generated singlet oxygen and dye sensitized photooxidation of amino acids known to be susceptible to dye sensitized photooxidation was made which showed the products to be identical. The photooxidation of lysozyme sensitized by hematoporphyrin was shown by application of tests involving deuterium oxide enhancement and azide inhibition to be mediated mainly by singlet oxygen. Studies of the effect of gas phase singlet oxygen on solid phase lysozyme and ribonuclease were used to further clarify the mechanisms of dye sensitized photooxidation and the effect of a potential pollutant on protein structures.

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The encouragement and support of my wife, Lee Ann, are also gratefully acknowledged. I would like to dedicate this thesis to her.

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ABSTRACT

The effects of chemically and/or microwave discharge generated singlet oxygen on proteins and their constituents were shown to parallel the effects of dye sensitized photooxidation. This supports the contention that dye sensitized photooxidation is mediated by singlet oxygen. Chromatographic comparison of the products of oxidation by microwave generated singlet oxygen and dye sensitized photooxidation of amino acids known to be susceptible to dye sensitized photooxidation was made which showed the products to be identical. The photooxidation of lysozyme sensitized by hematoporphyrin was shown by application of tests involving deuterium oxide enhancement and azide inhibition to be mediated mainly by singlet oxygen. Studies of the effect of gas phase singlet oxygen on solid phase lysozyme and ribonuclease were used to further clarify the mechanisms of dye sensitized photooxidation and the effect of a potential pollutant on protein structures.

INTRODUCTION

Raab (1900) reported, in 1900, that light, in the presence of sensitizing dyes, killed micro-organisms. This process was termed "photodynamic action". The effects on materials of biological interest include degradations of carbohydrates, nucleic acids, lipids and proteins. A number of "non-biological" organic compounds, generally containing an olefinic structure, also have been shown to be susceptible to photodynamic action. This effect is now frequently called "dye sensitized photooxidation", since it usually involves the oxidation of the target molecule. The term "photodynamic action" is reserved by some (but not all) authors for the pathological gross biological effects of sensitized photooxidation.

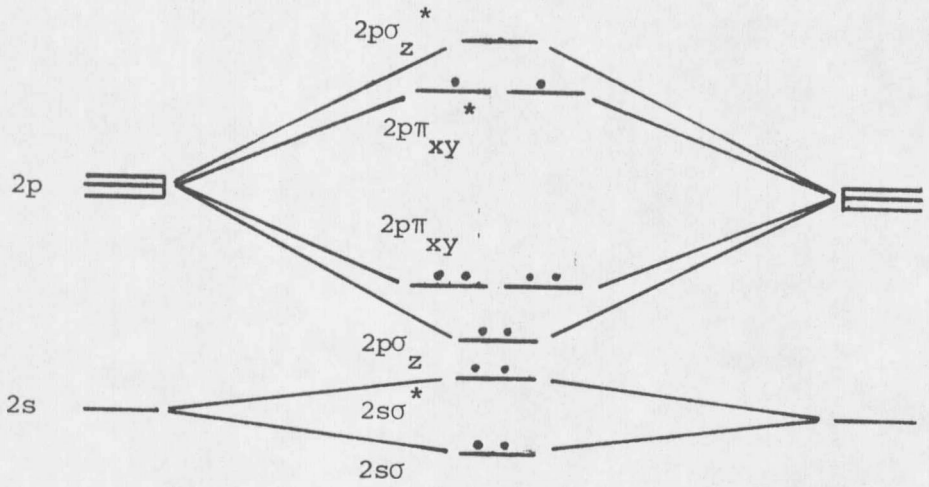
A number of different mechanisms have been proposed to account for photodynamic action. Until fairly recently, the most generally accepted views that the dyes absorbed light to give an excited dye electronic state. This either reacts with substrate to abstract a hydrogen atom or electron, thereby oxidizing the substrate and generating a reduced dye which may or may not react with molecular oxygen to regenerate the original dye or the excited dye may react with oxygen to form a dye-oxygen complex (also called a moloxide (Gollnick, 1968)) which can react to oxidize a receptor.

The hydrogen atom or electron abstraction mechanism has been demonstrated for some non-biological substrates, usually in the

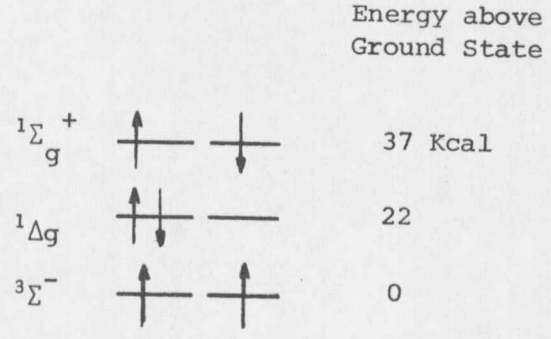
absence of oxygen. This mechanism is now often referred to as a Type I sensitized photooxidation. While it is very likely some rather specific photooxidations of biological compounds may involve this mechanism or something like it, this mechanism has not been proven for the oxidation of any biological compounds, especially proteins. Clear examples of the moloxide mechanism have not been demonstrated.

A major problem arose with these direct dye mechanisms. Dye sensitized photooxidations of certain olefinic compounds give more than one product in fairly consistent ratios. Generally, the ratios are not greatly affected by the species of the dye, even though the photosensitizers vary greatly in structure.

Recently, a mechanism originally proposed by Kautsky (1939), in 1931, has gained support and general acceptance to account for most dye sensitized photooxidations. He proposed that light is absorbed by the dye to give the dye electronic triplet which interacts with ground state triplet molecular oxygen to give a singlet molecular species, which is then the active agent in the oxidation. This is often referred to as a Type II sensitized photooxidation. His views were largely ignored for 25 years, despite demonstrations that the dye and the substrate in a sensitized photooxidation could be separated some distance by a layer impermeable to either but not to oxygen. He also showed that reactions could take place when dye and



a. Diagram of the Ground State



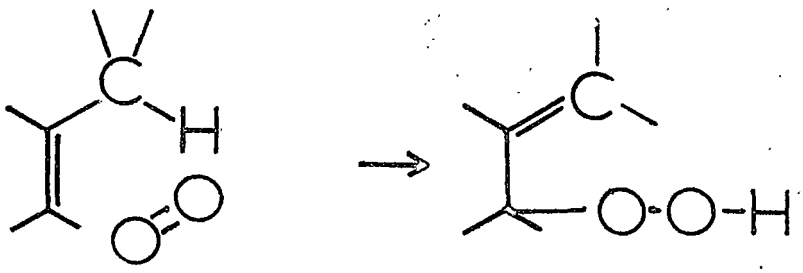
b. π_{xy}^{*} Orbitals showing Ground and Excited States

Figure 1. Molecular electronic state of oxygen.

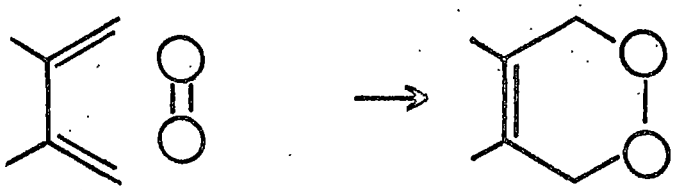
sensitizer were absorbed on separate grains of silica gel, allowing only for transfer of excited oxygen between them.

Figure 1a diagrams the molecular orbitals and the ground state triplet electronic configuration for molecular oxygen. The highest occupied orbitals are the antibonding degenerate π^*_{xy}, which are half filled. Figure 1b diagrams the electronic structure of these highest occupied orbitals in the ground triplet and the first two excited singlets. The $^1\Delta_g$ singlet is generally considered to be the main mediator in dye sensitized photooxidations. The $^1\Sigma_g$ state is thought to be easily inactivated and hence not easily available for reaction even in systems where it may be generated. A growing body of evidence supports Kautsky's mechanism

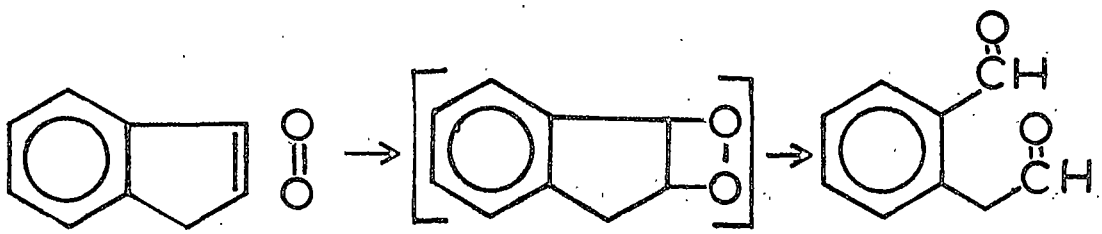
It is possible to generate singlet oxygen, especially the $^1\Delta_g$ state, by a number of methods which are independent of dye and light. When oxygen so generated is allowed to react with olefins which give distinctive ratios of products with dye sensitized photooxidation, the same products in approximately the same ratios are obtained. Dye free systems which can be used to generate singlet oxygen include electrodeless radio frequency discharges in low pressure oxygen, the reaction between hydrogen peroxide and "positive valence" halogen compounds, most commonly hypochlorite, the decomposition of transannular linear polycylic aromatic peroxides, the decomposition of triphenyl phosphite-ozone adducts, the decomposition of



1,3-Addition Forming a Hydroperoxide



1,4-Addition Forming an Endoperoxide



1,2-Addition Forming a 1,2-Dioxetane
Which Decomposes to a Dicarbonyl

Figure 2. Reactions of $^1\Delta_g$ Molecular Singlet Oxygen with Olefins

peroxychromates, the decomposition of peroxyacetyl nitrate (PAN) and, perhaps, certain enzymatic reactions.

Oxidations mediated by singlet oxygen typically are rather selective in the type of structures attacked and specific in the types of products obtained. Considerable parallel has been drawn between reactions of singlet oxygen and the Diels-Alder and "ene" reactions. Ground state oxygen behaves chemically as a diradical since it has unpaired electrons in its highest orbitals. The sigma singlet is also thought to react similarly since its electrons are also unpaired, but because of its short lifetime, its chemistry is poorly developed (Foote, 1968; Kearns, 1971; Ogryzlo, 1970). Since the delta singlet has all paired electrons, it has symmetry similar to ethylene and reacts with conjugated dienes and substituted double bonds in a similar concerted manner. Figure 2 illustrates the typical reaction course and products for these reactions.

Some instances of 1,2-addition to double bonds are known although this does not seem to be the typical course in most cases (Fenical et al., 1969).

The oxidation of indene shown in Figure 2 illustrates this type of reaction. This type of addition seems favored by electron rich substitutes on the double bond and a lack of α-hydrogens. In the case of 1,3-addition to an isolated double bond, reactivity is in the order of unsubstituted increasing up to tetrasubstituted by a factor of ten

for each methyl or equivalent. If substituents on the double bond are all different and each has the alpha hydrogen as shown in Figure 2, then generally all of the four different hydroperoxides will be produced in ratios which are fairly constant from one source of singlet oxygen to another. Production of such typical ratios is considered one of the more definitive tests of the presence of singlet oxygen in a given reaction system. While the same products may be obtained by certain free radical oxidations, the product ratios will generally be different and the whole reaction can be quenched by radical scavengers, such as substituted phenols, which is not the case with a single oxygen reaction (Foote, 1968).

Quenchers for singlet oxygen are known. The exact nature of their reaction is not clearly worked out, and is undoubtedly different for each type. The main types of quenchers currently considered are polyenes of seven or more conjugated double bonds, tertiary amines, azide ion and hydroxyl compounds. The most commonly cited example of the first is β-carotene with nine conjugated double bonds. A number of tertiary amines have been used in various studies, but the most commonly discussed one is 1,4-diazabicyclo[2.2.2.]octane (DABCO, triethylenediamine) (Kearns, 1971). Water is perhaps the best hydroxylic quencher. Work by Nilsson et al. (1972, 1973; Merkel et al. (1972)) has shown that the efficacy of hydroxyl as a quencher correlates with how well "tuned" the bond stretching overtone vibrations are to the

23 kcal. energy level of the singlet oxygen. Based on this observation, they devised an elegant test for the action of singlet oxygen in aqueous systems. Deuterium is enough more massive than hydrogen that bond vibration energies are "detuned" to a great enough degree that the lifetime of singlet oxygen in deuterium oxide is about ten times (20 μseconds vs. 2 μseconds) that in water. As a result, a reaction run in deuterium oxide, everything else being equal, will proceed up to ten times as fast because of higher steady state concentrations of singlet oxygen. Coupled with a parallel reaction with a quencher, such as azide, where singlet oxygen is inhibited, this constitutes the best current test of an oxidation for singlet oxygen involvement where olefinic addition product ratios can not apply.

Direct detection of singlet oxygen can only be done spectroscopically. The transitition from the 23 kcal./mole level to ground state causes an emission at 1,280 nm. This can be used to monitor the concentration of the species in the gas phase. Unfortunately, this region of the spectrum is not convenient to work with, due to instrumentation requirements, so this band is not often used. Both the delta and sigma singlets are able to undergo a remarkable bimolecular emission process which can pool the energy from two molecules to give, in the case of the delta species, an emission at 634 nm and for the sigma 382 nm (Bader and Ogryzlo, 1964). The interaction of the two different species gives a band at 478 nm. The bimolecular delta

band at 634 nm is often used to monitor the concentration of the delta singlet in gas reactors. This technique of concentration monitoring has not been shown to be useful in liquid reaction systems, even though it was the chemiluminescense of the reaction of hydrogen peroxide and the hypochlorite ion (at 634 nm) which called attention to this reaction as a source of singlet oxygen. The emission may come from bubbles and not dissolved oxygen. Since the 634 nm emission is bimolecular in nature, it is dependent on the square of the concentration of the delta singlet. The unimolecular 1,280 nm emission is directly proportional.

While more technically tractable as a source of monitorable emission than the 1,280 nm band, the 634 nm band is still faint enough to require rather sensitive equipment, including monochromation, for use as a monitor due to the relatively long radiative lifetimes of the Δ species and its bimolecular complexes. It was observed that the coal tar dye violanthrone gives an intense red emission with singlet oxygen (Ogryzlo and Pearson, 1968). This reaction is due to the transfer of the electronic energy from two singlet molecules in a two stage simple quenching process, without other chemical reaction. This reaction has been used to monitor the output of an electric discharge (in this case a microwave induced discharge) singlet oxygen generator. The reaction is intense enough to be readily visible to the eye in a semidarkened room. As in the case of the 634 nm band discussed above,

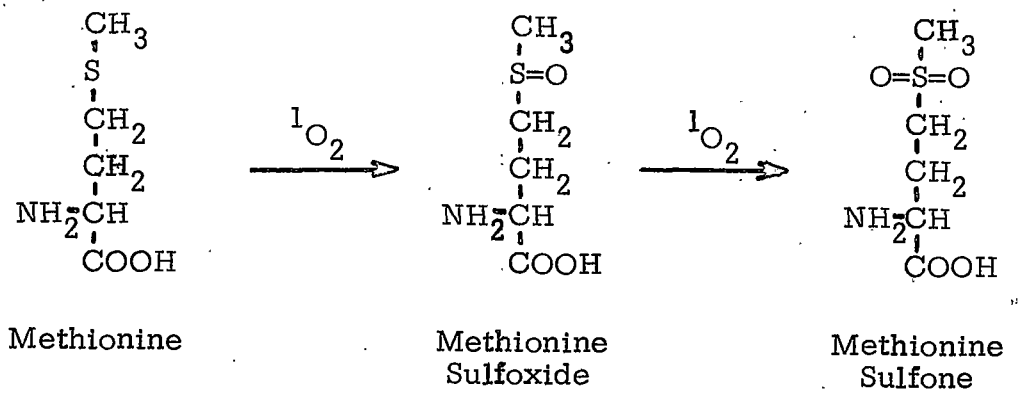
this luminescence is proportional to the square of the singlet oxygen concentration.

Photooxidation of substrates of biological interest, such as proteins and nucleic acids, is now generally considered to involve singlet oxygen, even though the picture here is somewhat less clear-cut than in the case of olefins. Sensitized photooxidation of nucleic acids seems to primarily attack the bases. While all the bases seem to be susceptible to photooxidation, guanine is the most susceptible of the five common ones. Adenine is the least reactive of this group, being about an order of magnitude less reactive than guanine. Studies of the singlet oxygen oxidation of nucleic acid components have demonstrated these relative reactivities using, besides sensitized photooxidation, radiofrequency discharge-flow systems and hydrogen peroxide-hypochlorite reactions (Clagett and Galen, 1971; Hallet et al., 1970). The main obstacle to clarification of what exactly is happening is these reactions is that the products from any of the bases are complex, poorly characterized mixtures, Of the studies mentioned, the one with the hydrogen peroxide-hypochlorite suffered from the added handicap of side reactions with the reagents, which have nothing to do with singlet oxygen. This has proven to be a problem with studies of this system and proteins.

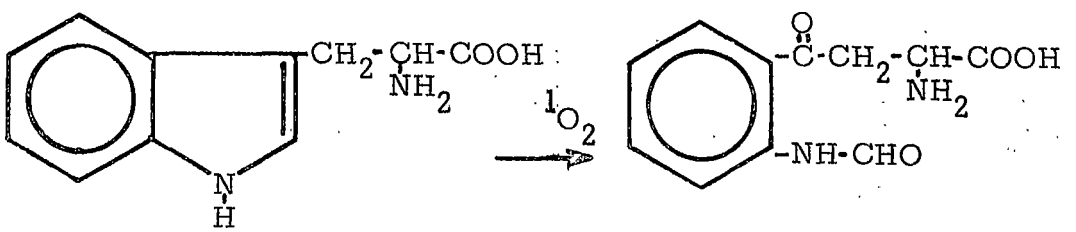
That photodynamic action on proteins can proceed by singlet oxygen pathways is well established. However, there is a considerable

body of evidence that in many, if not most, cases the reaction is not as simple as with olefins. There is evidence for dye-substrate interactions and the degree of protonation of the substrate may effect the reaction. Of the twenty or so amino acids found in proteins, only five have been demonstrated to be notably susceptible to sensitized photooxidation. These are methionine (Met), Histidine (His), Tryptophan (Trp), Tyrosine (Tyr) and Cysteine-cystine (CysH, Cys_2). That the first two are probably photooxidized by singlet oxygen in most of the sensitized systems studied is supported by recent studies using the deuterium oxide and azide test systems. These two residues are the usual targets of sensitized photooxidation of proteins and the inactivation of enzymes usually parallels their destruction, especially that of His. The same can be said about Trp with less confidence. The nature of the photooxidation of Tyr and Cys is far less clear and evidences for direct dye mechanisms exist.

The products of photooxidation of these amino acids are well characterized only for Met and fairly well understood for Trp. Met is oxidized initially to a sulfoxide and somewhat more slowly to the sulfone. There is one report of sensitized oxidation to methional by lumiflavin containing sensitizers, but there is considerable reason to believe that this may represent an example of a direct dye mechanism. Trp can be oxidized to several products. If the α-amino group is blocked by an amide bond, the main product is



Methionine | Methionine Sulfoxide | Methionine Sulfone



Tryptophan | N'-Formyl-Kynurenine

Figure 3. Products of photooxidation of methionine and tryptophan.

N´-formyl-N-blocked-kynurenine. If the amino is not blocked, besides the kynurenine product, melanine products are formed. These are usually intractable brown polymers. Other products have been observed under special conditions. There seems to be some slight question about the yield of kynurenine in the blocked case. Most authors feel it is quantitative, while others question this but report no other identifiable products. The photooxidation of Trp sensitized by lumiflavin and such lumiflavin containing compounds as riboflavin shows some features which seem to require invoking some non-singlet oxygen mechanism. Oxidations sensitized by the more usual rose bengal or methylene blue in oxygen rich media seem to have features consistent with singlet oxygen mechanisms. The lumiflavin sensitized photooxidation of Trp can be accelerated by the presence of roughly equimolar amounts of adenine. At the same time, oxygen was found to actually quench the reaction, giving lower quantum yields (Yoshimura and Kuto, 1973).

The oxidation of His is complex. A study by Tomita et al. (1969) reported the isolation of 17 compounds in pure form from the sensitized photooxidation of N-benzoylhistidine. They suggested a course of reactions which begins with a 1,4-cycloaddition of singlet oxygen to the imidazole ring.

The photooxidation of Cys, either as CysH or Cys_2, is less well understood. It is susceptible to photooxidation, but usually at a

much slower rate than Met or His. Jori et al. (1969a) and Bellin and Yankus (1968) have reported the selective photooxidation of Cys, especially as CysH, by crystal violet. This dye will not sensitize the photooxidation of His or Met. Work on model disulphide compounds with gas discharge generated singlet oxygen, supports the idea that Cys_2 should be susceptible to singlet oxygen attack. The only product so far identified for the sensitized photooxidation of Cys is cysteic acid, although the models suggest others should be produced, perhaps as intermediates.

The photooxidation of Tyr is perhaps the least understood of the five. Tyr is not very susceptible to oxidation in the uncharged form. At higher pHs, where the phenolic group is ionized, it does show some reactivity. This correlates with the expectation that singlet oxygen is electrophilic and would tend to react better with the ring when it has a higher electron density. A similar effect is noted with His. His is less reactive at low pH where the imidazole is protonated than at higher pHs where the ring does not have a positive charge. Very little is known about the products of Tyr photooxidation, which seem to be melanine type dark polymers.

There have been no reports of oxidations of amino acids as such by dye free singlet oxygen systems. Churakova et al. have reported the oxidation of lysozyme in a flow-electric discharge system. They demonstrated that the chromatographic behavior of the oxidized enzyme

matched that of methylene blue sensitized photooxidized lysozyme and that the disappearance of His and Typ paralleled that of the photo-oxidized reaction. They did not identify any amino acid products.

Effects that are so far not explained with regard to the photo-oxidation of amino acids, and even more so of proteins, are aspects of dye substrate specificities. In reactions with olefins, a test used for the singlet oxygen mechanism is that with different sensitizers, two different substrates will give fairly consistent ratios of the rates at which they are oxidized. This test has been extended to electric discharge and peroxide-hypochlorite systems with consistent results. When this type of comparison is made with a range of sensitizers on the amino acids listed above, the ratios are rarely consistent. In one study, the ratio of oxidation velocities V(Trp)/V(His) varies from 0.9 for eosine Y to 0.25 for thionine. Thionine is noted in one study with dimethylanthracene, as clearly being able to function in both Type I (electron abstraction) and Type II (singlet oxygen or Kautsky) mechanisms, depending on the availability of oxygen and substrate (Kramer and Maute, 1973). Several authors have shown that the decoloration or bleaching of methylene blue can be achieved under anaerobic conditions in the presence of amino acids (Weil, 1951; Knowles and Gurnan, 1972). A summary of the results of two different studies is that: 1. Trp reacts under these conditions at pH 5.8 and 8.0; 2. His reacts at pH 8.0 but not 5.8; 3. CysH reacts at pH 5.8;

4. At pH 5.8 Tyr, Cys_2, Met, Alanine, and Phenylalanine are not significantly reactive; 5. At pH 8.0 Met and Tyr may be slightly active; 6. N-acetyl-Trp does not react at pH 5.8; 7. The products of the amino acids under these conditions have not been identified. It is of interest to note in passing that methylene blue differs from thionine by four methyl groups (it is N,N,N´,N´-tetramethylthionine) and it would not be surprising if it could undergo electron abstraction reactions, and it is well known to be reduced by many active reductants. Photobleaching does not seem to take place at an appreciable rate in the presence of oxygen. If a photobleached methylene blue-amino acid solution is allowed to oxygenate, most of the methylene blue is regenerated.

A sensitizer, hematoporphyrin, which usually gives results with olefins consistent with the singlet oxygen mechanism, is reported to give anomalous results with amino acids (Jori et al., 1969b). At pH 6.1, Cys_2, His, and Tyr were found to be unreactive and Trp was only slightly. Under the same conditions, Met was rapidly and quantitatively converted to Met sulfoxide. The slight reactivity of Trp was removed when it was blocked at the amino group. The same authors reported that under these conditions one of the two Met residues in lysozyme can be oxidized to the sulfoxide, leaving all other residues untouched. A later paper in the same series reported that two molecules of hematoporphyrin were bound per molecule of lysozyme and that

this binding shifted the absorbtion spectrum of the dye (Jori et al., 1971). They reported that light absorbed by the bound dye and not that absorbed by the unbound was effective in causing the photooxidation.

When an intact enzyme is subjected to dye sensitized photooxidation using, most commonly, methylene blue, rose bengal or proflavin, considerable selectivity in which residues are oxidized is noted. The results can usually be interpreted in terms of availability of the target residue to the singlet oxygen. An occasional case is noted, as with lysozyme and hematoporphyrin where the results suggest that the reason one group is selected over another is some type of binding of the dye to a specific site on the protein, which, even if the oxidation is singlet oxygen mediated, would permit a proximity effect. Generally, if a protein is unfolded by use of high molarities of urea, etc., all the His and most of the Trp can be oxidized (Sajgo, 1963). Most of the Met is probably also oxidized under the same circumstances, but the usual work up involves acid hydrolysis with subsequent automatic amino acid analysis. This generally will show only the Met, as MetSO is usually reduced to Met by acid hydrolysis with hydrochloric acid. Basic hydrolysis is necessary to recover the sulfoxide.

Considerable use has been made of these types of selective photooxidations to define residues, particularly those of His, which are "critical", usually implying in the active site, to the action of

an enzyme. Considerable care has to be used in interpreting this data, since in the reported case of oxidation of one residue of Met in lysozyme (Jori et al., 1969b), the residue is reported to be Met-12. In X-ray determined structures of lysozyme, this residue is on the opposite side of the enzyme from the active site. The conversion of this Met to the sulfoxide is reported to lower the activity to 54% of that of the native enzyme. The implication of this is that the addition of one atom of oxygen to the Met can so alter the conformation of the enzyme that its catalytic activity is largely hindered, even though this residue is not directly involved in the catalysis. Much of the older literature in this area report effects which can be interpreted as either destruction of "catalytic" residues or conformational changes. In most cases, the former interpretation was, at least, implied. In studies where substrate or enzyme cofactors can be shown to protect the enzyme from sensitized photooxidation, the interpretation that the target residues are catalytic or allosteric sites is on firmer ground. One author (Westhead, 1972) has suggested that the use of dye sensitized photooxidation to investigate protein structure has not yet fulfilled its earlier promise.

STATEMENT OF PURPOSE

This work was initiated to elucidate the mechanisms of the dye sensitized photooxidation of proteins. This class of photooxidations has value for the selective modification of proteins to investigate their nature of action. Usually, an investigation of this type will be considering the mechanism of catalysis of an enzyme. By selectively modifying some or all of the protein residues susceptible to sensitized photooxidation--His, Met, Trp, Cys and Tyr--information can be learned about the involvement of these residues in the catalysis. The better the sensitized photooxidation process is understood, the more useful will be the information obtained from these photooxidations. If highly specific mechanisms of action could be found, ones which oxidized only one type of residue, then the utility of dye sensitized photooxidation could be improved as the involvement of single residues or types of residues would be open to investigation.

At the time these studies were started, it was generally felt that, in at least some cases, dye sensitized photooxidations could be mediated by singlet oxygen in Type II mechanism. However, no data were available which could be explained only in terms of a Type II mechanism, to the exclusion of Type I mechanisms involving direct dye interactions with the substrates.

It was known that singlet oxygen could be generated by dye free systems, and that oxidation of certain olefinic substrates by these

systems gave the same distinctive products that were observed in dye sensitized photooxidations. However, it was not known if dye free singlet oxygen oxidations of proteins and amino acids gave the same products that were observed in dye sensitized photooxidations. Indeed, it was not even known if amino acids would react with dye free singlet oxygen.

The first major question, then, that was addressed in this investigation was: Does dye free singlet oxygen react with proteins and amino acids and, if so, do the amino acid products of dye free singlet oxygen oxidation compare with those of dye sensitized photooxidation? The study of this question investigated the inactivation, in solution, of lysozyme by microwave discharge generated singlet oxygen, but primarily investigated the reactions of the amino acid Met and the amides, N-benzoylhistidine, N-acetyltryptophan and N-glycyltryptophan with both dye sensitized photooxidation and microwave discharge generated singlet oxygen. The results of the oxidations of the amino acid and amides were compared by thin layer chromatography.

Subsequent to the time these investigations were performed, Churakova et al. (1973) reported on their work in deactivating lysozyme in solution by microwave generated singlet oxygen. While they did not investigate the nature of the amino acid products, they did show that the disappearance of Trp and His paralleled that observed in methylene blue sensitized photooxidation. This answered most of

the major remaining questions regarding the reactivity of an intact protein in this type of system so other areas of the mechanism of dye sensitized photooxidation were investigated.

The second major question that was considered was: Are there any truly specific dye sensitized photooxidations that could be used for highly selective modification of individual residues or types of residues to clarify the residue's purpose in the protein? Since a Type II, singlet oxygen mediated, mechanism would be expected to react with all the susceptible residues, at least to some degree as they are available to the mediator, this was, then, the search for a dye reacting by a Type I mechanism where specific dye-substrate interactions would provide selectivity. At least two systems had been reported in the literature where this type of selectivity had been observed. The first, reported by Bellin and Yankus (1968) and Jori et al. (1969a), involved the specific photooxidation of cysteine sensitized by crystal violet. The second, reported by Jori et al. (1969b), involved the specific photooxidation of Met, both free and in the protein lysozyme, sensitized by hematoporphyrin. These systems were investigated (the first very briefly) to confirm the reports so that the work might be extended to provide useful protein modification tools. The first reaction was studied with a Clark oxygen electrode. The methods of Nilsson, Merkel and Kearns (1972), involving deuterium oxide enhancement and azide quenching of singlet oxygen to

differentiate Type I from Type II mechanisms, were used to investigate the second.

The effects of singlet oxygen in the gas phase on solid proteins and amino acids were investigated. This work was an outgrowth of the work with proteins and amino acids in solution using microwave discharge generated singlet oxygen, but offered the opportunity to learn something about the effect of a potential air pollutant on protein structures. Work by Davidson and Abrahamson (1972) and from the laboratory of Pitts (Coomber et al., 1970) have shown that singlet oxygen can be generated in polluted atmospheres by photoreactions sensitized by various pollutants, making this investigation of something more than academic interest.

METHODS AND MATERIALS

Gas Chromatography

All gas or paper phase chromatographic determinations were run on a F & M Biomedical Gas Chromatograph Model 400. Two columns were used. Both were filled with an absorbant prepared by coating 45 g of Chromosorb W with 5 g of Carbowax 20 M in 100 ml of chloroform, then removing the chloroform by low pressure evaporation and heating overnight in a low temperature oven. A four foot long column of silanized ¼ inch O.D. glass and an 18 foot column of ¼ O.D. copper tubing were made. The short glass column was used in initial studies with 2,3-dimethyl-2-butene and its oxidation products. The longer column proved more satisfactory and was used with both olefins and their products. Chromatograms were run at various temperatures up to 200°C with most work being done at 160°C. Flame ionization detection was used with helium carrier gas.

Mass Spectrometry

Mass spectra was taken on a Varian CH-5 Mass Spectrograph operated by Dr. P. W. Jennings using a Varian Aerograph gas chromatograph inlet.

Visible and Ultraviolet Spectrophotometry

Absorbtion spectra were recorded on a Cary-14 Recording Spectrophotometer. Initial work with lysozyme activity assays used

Beckman DB or B. and L. Spectronic 20 spectrophotometers. Later work, which was the majority, and all ribonuclease activity assays used a Beckman DU-2 spectrophotometer. Determinations of the ratio of the absorbtion at various wavelengths were done on the DU-2.

Automatic Amino Acid Analysis

All analyses were run on a Beckman Model 120 C Amino Acid Analyzer equipped with an Infotronics integrator. Three columns were used in these studies. They were about 6 cm, 21 cm and 60 cm long. The first two were used for resolution of basic amino acids using pH 5.26, 0.4M Na^+ citrate buffer, while the long column was used for acidic and neutral residues using two buffers which automatically changed during a run. These buffers were pH 3.28 and 4.25 citrate, both 0.2M Na^+. Samples of approximately 1.0 ml were introduced to the columns by means of automatic metering values. A "good" loading of a particular amino acid is 0.02-0.3 μmole/ml. Outputs were both in the form of strip chart absorbance graphs and an integrated number which gave relative values for the peak areas. Calculation of the results from these outputs consisted of dividing the unknown's integration for a particular peak by the integration for the same residue in the most recent standard run. This number was then multiplied by the number of moles, usually 0.1 μmole, that the standard represented. When all residues of a run were converted to this molar value, a

common denominator division converted the molar values to numbers of residues. Since all proteins used in this study are well characterized as to their integral contents of various residues and the relative amounts of phenylalanine (Phe), tyrosine (Tyr), alanine (Ala), aspartic acid (Asp) and lysine (Lys) are relatively less likely to be affected by artifacts of the determination, one or more of these residues were generally used as a basis for a common denominator calculation.

Microwave Electrodeless Discharge Gas Flow Singlet Oxygen Generator and Reactor

The reactor was constructed of the following materials and equipment. The power source was a Raytheon PGM-10-X2 microwave power generator operating at 2450 MHz. This instrument seems to have been originally designed for medical therapeutic purposes. Figure 4 shows the form and dimensions of the glass part of the system. The central discharge tube was of Vycor fused quartz glass. The rest of the glassware was Pyrex or Kimax. The Vycor tube was necessary because of thermal stresses in the vicinity of the discharge. The power from the generator was coupled to the gas stream in the discharge tube by means of either an Evanson Cavity or a Type C Raytheon medical antenna as modified at the suggestion of Dr. Louis Owen (1973). The Evanson Cavity was used in early work, while the Owen-Type C proved more satisfactory and was used later. The Owen modification consisted of

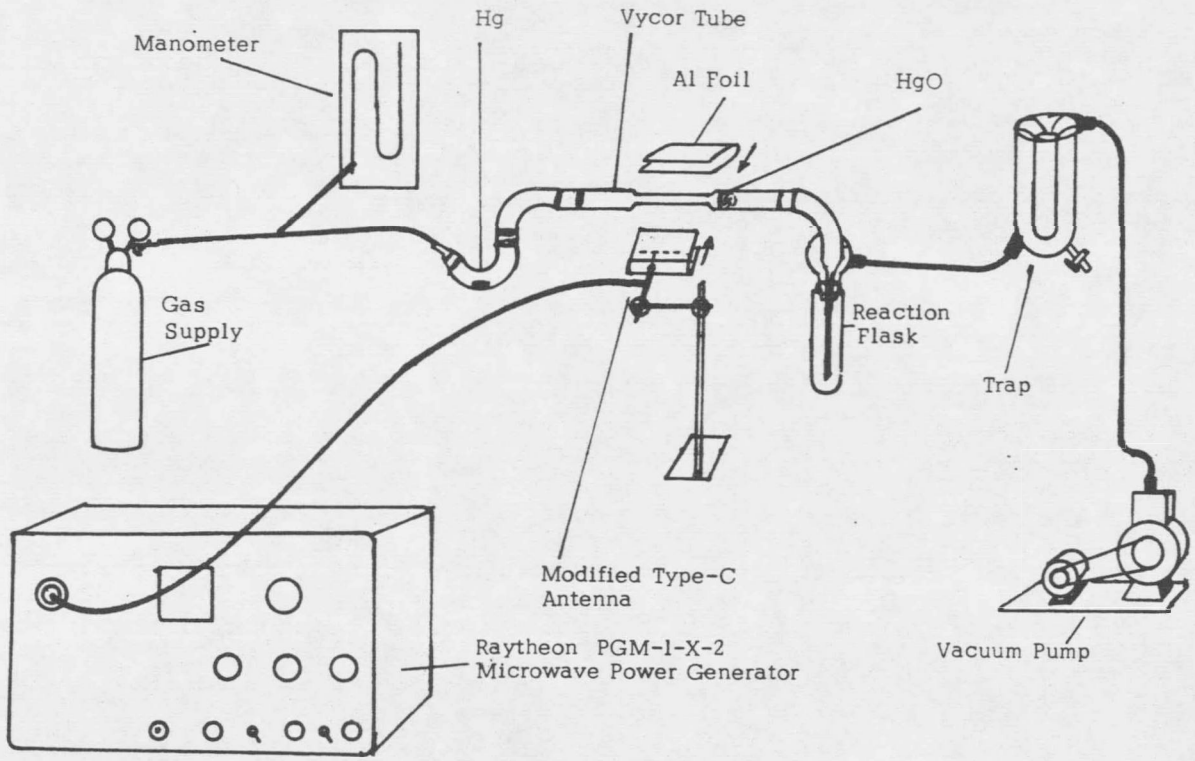


Figure 4. Microwave discharge singlet oxygen generator.

bending the reflector wings of the Type C antenna from their initial right angle until the sides are parallel and about 35 mm apart. A sheet of aluminum foil was placed across the open side to give a rectangular box, open at both ends, of 35 mm by 62 mm cross section. The protective plastic shield over the dipole was removed as it tended to melt and was not needed for protective purposes. Cooling of the discharge zone was provided by jets of filtered house air. The Evanson Cavity has provision for attachment of air jets. The modified Type C was cooled by directing air on the dipole. A vigorous air jet is necessary.

A mixture of 10% oxygen in helium (Airco Rare and Specialty Gases) was used in the oxidations in liquid solutions. Other work used pure oxygen or carbon dioxide. The helium diluted oxygen was necessary when working with aqueous solvents to prevent excessive evaporation. This reactor will not maintain a discharge at pressure much above 10-15 torr in pure oxygen. The discharge can be maintained up to 50-60 torr in the diluted gas so that water loss from the reactor by evaporation at low pressure can be minimized. The pressure was usually maintained at 15-20 torr during reactions with liquids. The power output of the generator was rated at 100 watts. Operation was at 20 to 80% power. A discharge from a Tesla coil was used to start the discharge. There was no provision available for monitoring either gas flow rates or singlet oxygen concentrations directly.

There were indications from later work using violanthrone to monitor singlet oxygen output that optimum conditions were 15 torr at 20% power in 10% oxygen in helium and three torr at 20% power in pure oxygen.

The gas stream passed over a small pool of mercury ahead of the discharge. A ring of mercuric oxide formed downstream from the discharge. This is supposed to remove atomic oxygen by surface catalyzed recombination and prevent its formation of ozone (Furukawa et al., 1970). During start ups after the discharge tube had been cleansed (rinses of 3 N nitric acid), the tube holding the mercury was heated briefly with a match to rapidly form the oxide ring before substrate was placed in the reaction flask.

The vacuum pump was protected by a dry ice trap (liquid nitrogen with methanol reactions) to remove water evaporated from liquid reactions. Due to evaporation from aqueous solutions, 10-20 ml per hour of water was added to maintain the volume. The solutions were maintained at 0-5°C. Because of evaporative cooling, it was necessary to use a water bath of cool (ca. 10-15°) water to warm the reaction flask sufficiently to prevent freezing.

Reactions which used solid substrates and contained no liquids did not use a cold trap. When the substrate was present as or on a powder, then a glass lypholyzer assembly was used as a dust trap to protect the pump. The gas stream entered the lypholyzer at the

bottom, through one of the ports provided for attachment of samples to be lyophilized, and exited at the top permitting dust opportunity to settle.

Instructions for Operation of the Reactor

1. Assemble the reactor as shown in the figure. Don't place substrate in initially. Be sure small (5 mm diameter or greater) pool of mercury is in the mercury source. Use only Vycor or equivalent in the discharge tube due to thermal stress. Pyrex won't work.

2. Align the dipole antenna of the modified Type-C antenna parallel to the Vycor tube as far upstream on the narrow part of the tube as possible. The dipole should be as close (1-2 mm) as possible without touching. Cover the open side of the antenna with aluminum foil.

3. Align the air jets to blow, as nearly as possible, on the dipole and the part of the tube next to it. Turn on enough air that it seems noisy. The jet can be conveniently constructed from a plastic 2 × 15 cm drying tube fitted with end caps which have tapered nipples for attachment to gas hoses. Tape the caps on. A disc of thin, open fine pore sponge rubber in the tube makes a good low-pressure-drop filter. Use the nipple at one end for the jet and attach the other to the air line.

4. Make sure that all connectors for the power unit's coaxial cable are tight. The "Range" setting should be at "CW" and the "Radiate" switch down. Turn on the "Preheat" switch. If the "Standby" light does not come on in 2-3 minutes, recheck the cable connections at both the antenna and the power unit. There is a safety interlock on the unit which prevents the "Standby" from coming on if the cable circuits are not closed. If the power is turned on when the antenna is not properly connected so that the power can be radiated, damage will occur to the main microwave power supply magnetron.

5. Evacuate the system and check for air leaks. With the pressure as low as possible (less than five torr), turn on the "Radiate" switch. The red light should come on. Adjust to between 20-80% power. Do not operate over 100%. Rarely, a discharge will be noticed in the tube at this point. If not, bring a Tesla coil (or other high voltage corona discharge source), adjusted to give a slight corona, near the Vycor tube. (Touch the tube if necessary). Don't ground a spark into the antenna. If a discharge doesn't start, then check the power setting (the meter sticks: tap on it) and the antenna alignment. Turn off the "Radiate" before adjusting the antenna. Radio-frequency burns are supposed to be nearly painless and bloodless, but rather smoky and smelly from pyrolyzed protein (Owen, 1973). When a discharge starts, a dip in the "percent power" reading of the meter

will be noted. The larger the dip, the better the power is coupling to the discharge. This quality of coupling does not seem too important, except that it is easier to keep a discharge going with good coupling, as any discharge whatsoever seems to produce about the same amount of singlet oxygen. While the heat output and stray microwave fields are less at 20% power, the discharge is less stable there than at 40%. There does not seem to be any reason to operate it at higher settings. If a discharge will not keep going at 40% power, at pressures less than 10 torr, check the antenna alignment and the cleanliness of the inside of the Vycor tube. Grease or too much mercuric oxide deposit can interfere with the discharge.

6. Adjust the gas source to give about three torr with oxygen or 20 torr with 10% oxygen in helium.

7. On initial start up, when no mercuric oxide ring has formed, heat the mercury source with a small flame (match) until a yellowish film is noted downstream from the discharge. A smaller ring will form upstream.

8. At this point, the discharge will be blue if very pure oxygen is used. Traces of nitrogen, either from the tank or from air leaks, will give it a pink color. If a yellowish glow (dark room) is noted downstream from the discharge, distill more mercury by heating

until the glow leaves. The yellow glow seems to be due to a reaction between traces of atomic oxygen and traces of nitrogen oxide, NO, and is very sensitive according to the literature (Bader and Ogryzlo, 1964). It may be necessary to check for this and reheat the mercury from time to time on long reactions.

9. Once an oxide ring has formed, turn off the "Radiate" switch and release the vacuum. Place dry ice-acetone mixture in the trap if a liquid substrate is used. Seal the substrate in the reaction flask, either the 3 × 20 cm tube or the 500 ml lypholyzer flask depending on volume requirements, and repeat the start up procedure.

10. After a few hours of operation, mercuric oxide deposits will become thick enough to interfere and should be removed with 3N nitric acid rinses. Reform the initial HgO film before exposing substrate.

11. When using water solutions at 20 torr, the low pressure evaporation may cause ice formation in the reaction flask. A beaker of cool water as a bath will prevent this. It is usually necessary to make up evaporation losses every half hour or so. Ice deposits in the dry ice trap will eventually prevent further operation until they are removed.

12. When using dry reactions, a tuning spot of a small amount (0.1 ml of a 5 mg/50 ml solution of chloroform) of violanthrone on filter paper taped to the inside of the reaction flask is useful for adjustment of pressure and power. Singlet oxygen output is maximized at the brightest red glow.

Oxidation of Model Olefins by Microwave Generated Singlet Oxygen

2,3-Dimethyl-2-butene (tetramethylethylene, TME) and 2-methyl-2-pentene (2M2P), (both obtained from J. T. Baker, "Baker Grade"), were oxidized in methanol using the microwave reactor. The following reaction conditions were used: Two to four grams of the olefin were were dissolved in 30 to 40 ml of methanol. The reaction flask was cooled in a dry ice-acetone bath. The trap, which used dry ice in the aqueous operations, was charged with liquid nitrogen. The samples were oxidized for from two (TME) to four hours. The specific operations are described below.

A. 2,3-Dimethylbutene (TME): Two g (0.0238 mole) of TME was added to 30 ml to methanol and oxidized for 105 minutes. The solvent was evaporated on a steam bath until about 5 ml remained. The solution was cooled in an ice bath and 0.7 g (0.0186 mole) of sodium borohydride was added to reduce any hydroperoxides to the corresponding alcohols. The preparation was allowed to warm slowly and sat overnight at room temperature. Fifteen ml of methanol was added and the

solid residue was filtered out. Samples were analyzed by vapor phase chromatography (VPC) and mass spectrometry.

B. 2-Methyl-2-pentene (2M2P): Two g (0.0238 mole) of 2M2P was added to 27 ml of methanol. The sample was oxidized for 4.5 hours. After oxidation, the solution was cooled in a dry ice bath and 0.5 g (0.0132 mole) of sodium borohydride was added. The solution was kept cold overnight in a dry ice bath then allowed to warm slowly. Sediment was filtered off. Samples were analyzed by VPC and mass spectrometry.

C. 2-Methyl-2-pentene: Four g (0.0476 mole) of 2M2P in 40 ml of methanol was oxidized for four hours. The methanol solution was evaporated to a volume of about 2 ml using 40° water bath and vacuum. 1.2 g (0.0045 mole) of triphenyl phosphine was added in about 50 ml of diethyl ether to reduce peroxides. Samples of this material were analyzed by VPC.

Dye Sensitized Photooxidation of Model Olefins

The same two compounds that were oxidized in the preceding section were photooxidized using rose bengal as a sensitizer in order to provide samples of product alcohols for comparison. Illumination was provided at a distance of about 15 cm by a 150 watt G.E. spot lamp. The samples were placed in a 150 ml erlenmeyer flask which was flushed

with pure oxygen in dim light and connected to a one liter gas buret filled with water vapor saturated oxygen. The flask was cooled in a water bath consisting of a 500 ml beaker filled with water and a copper coil which was immersed in the water and connected to the tap water line. Temperature were maintained at 25-30°C. The samples were stirred magnetically. Illumination was through the side of the flask. The course of photooxidation was followed by oxygen consumption. When oxygen consumption had essentially stopped, the solutions were poured through a three cm bed of A-25 DEAE Sephadex. The peroxides were reduced with sodium borohydride. Mass spectral and VPC analyses were made of the solutions. In the case of 2M2P, special attention was paid to the product ratios of the product alcohols. An authentic commercial sample of 2-methyl-1-pentene-3-ol (Alfred Bader Chemicals) was analyzed by the same techniques.

Specific reaction conditions were as follows:

A.1. 2,3-Dimethylbutene (TME): One g (0.0119 mole) of TME was mixed in 30 ml of methanol with 10 mg of rose bengal. The solution consumed 354 ml of oxygen at 25° and 640 torr (0.0124 mole) in two hours with no more than two ml variation in the next half hour. Sodium borohydride (0.45 g, 0.019 mole) was added, after removal of the dye, and the solution was allowed to stand overnight. The only analysis made of this preparation was by VPC.

2. One gram of TME (0.0119 mole) was dissolved in 20 ml of methanol with 10 mg of rose bengal. The solution consumed 330 ml of oxygen at 650 torr and 25° (0.0115 mole). The dye was removed and 0.45 g (0.019 mole) of sodium borohydride was added. The solution was not cooled before adding the reductant and about a third of the solution was lost due to overly vigorous reaction. The solution was neutralized with hydrochloric acid, filtered and distilled. Most of the liquid distilled between 61 and 70°, taken in several fractions. Most of the product seemed to be in a 0.3 ml fraction taken at 84-86°, just before dryness. This material was used for comparison with the products of the microwave oxidations. VPC and mass spectral analyses were performed on this material

B.1. 2-Methyl-2-pentene: Two grams of 2M2P (0.0238 mole) was mixed with 20 ml of methanol and 10 mg of rose bengal. The solution consumed 690 ml of oxygen in 13 hours at 640 torr and 25° (0.0221 mole). The dye was removed. The solution was not cooled further and when 0.9 g (0.038 mole) of sodium borohydride was added, it foamed up and about half was lost. The remainder was refrigerated overnight, filtered and analyzed by VPC and mass spectrometry.

Results of Oxidation of Model Olefin Compounds by Microwave Generated Singlet Oxygen and Comparison to Dye Sensitized Photooxidation

The sole goal of this block of work was to determine if the microwave discharge reactor was indeed generating singlet oxygen. Two model olefins were used, 2,3-dimethyl-2-butene (tetramethylethylene, TME) and 2-methyl-e-pentene (2M2P). The TME is not a good choice for a study of this type as it gives only one product with singlet oxygen, 2,3-dimethyl-3-hydroperoxy-1-butene. The same product can be generated by free radical mechanisms (Foote, 1968). The 2M2P offers the advantage that, while less reactive than TME, it is more diagnostic. It yields two products, 2-methyl-3-hydroperoxy-1-pentene and 4-methyl-4-hydroperoxy-2-pentene which are formed in nearly equal amounts in singlet oxygen reactions. The hydroperoxides are reduced to the corresponding alcohol for analytical purposes.

In the first oxidation of TME by rose bengal sensitized photooxidation (as described in the preceding section) after an equimolar amount of oxygen had been consumed, oxygen consumption ceased. A gas chromatographic determination of the product, after reduction and removal of the dye, showed only one peak appearing much after both the starting material and the methanol solvent.

In the second photooxidation of TME, again an equimolar amount of oxygen was consumed with little further detectable consumption. The gas chromatographic determination of the product alcohol matched

that of the first oxidation. A mass spectrum of this was interpreted as being consistent with the expected product.

When TME was oxidized in the microwave reactor, yields were low (estimated as 10% or less of the starting material) but the only product observed matched the photooxidation product both gas chromatographically and mass spectrally.

The results of the comparison oxidation of 2M2P supported the presence of singlet oxygen in the microwave reactor. The only problem that might be open to question is the product ratios. Two product alcohols were observed in both types of oxidation. In the case of the photooxidation, the ratio of the first to the second peak from the VPC was 1:1.05, which is very near the value reported in the literature (Foote and Denny, 1971). The ratio was 1:1.16 to 1:1.20 with the microwave reactor. I cannot definitely account for the difference. I can note, however, that the more volatile (first chromatographic peak) product is in lowest yield, which may be a factor in this type of low pressure liquid reaction system. Foote and Denny did note variances in the ratio of "up to 20%" in their study of the effect of different solvents on the dye sensitized photooxidation of 2M2P and Peters et al. (1972) noted a ratio of 1:1.13 for the reaction with potassium peroxychromate generated singlet oxygen.

The mass spectra of the products from both reaction systems matched for their respective peaks. The spectra were assessed as

being consistent with the expected products. The first peak from the gas chromatograph was assigned to 4-methyl-4-hydroxy-2-pentene and the second to 2-methyl-3-hydroxy-1-pentene. An authentic sample of the latter proved to have the same mass spectrum.

The evidence supports the conclusion that the microwave reactor generates singlet oxygen. No other products were noted. Cleavage products would be expected if ozone were a significant product of the reactor.

Hydrolysis of Proteins in Preparation for Automatic Amino Acid Analysis

Both acidic and basic catalyzed hydrolyses of proteins were used in these studies. Acid hydrolysis used either six N hydrochloric acid or two N methanesulfonic acid with deoxygenated samples. Methanesulfonic acid has been shown to give superior Trp recovery. Basic hydrolysis used 3.3 N sodium hydroxide. The following procedures were used with any important exceptions noted in descriptions of individual experiments.

A.1. Acid hydrolysis with hydrochloric acid. One ml of the protein solution, generally containing 1-4 mg of protein in distilled water, was placed in a freshly cleaned and heat dried Carius tube or necked down 20 ml ignition tube. One ml of 12 N hydrochloric acid was added. The lower end of the tube was placed in a dry ice-acetone

bath to freeze the contents. It was then evacuated by means of a vacuum pump and, while still under vacuum, warmed slightly until melted. This melting was usually accompanied by swirling on a Vortex type mixer. If frozen before evacuation, foaming problems are minimized. Maintaining the vacuum, the sample was frozen and thawed with swirling twice more. The tube was sealed by drawing the neck out while heating with a natural gas-oxygen torch, all the while maintaining the vacuum. The samples were then placed in a sand bath in an oven or oil bath thermostat at the temperatures and for the times noted, usually 108-110°C for about 24 hours. After heating the tubes were removed, cooled and opened by scoring with a file and breaking. The samples and washings were transferred by means of Pasteur pipettes to small beakers or bottles of 5-15 ml capacity and one or more cm mouth width (to provide a good free path for evaporation). The beaker was then desiccated under vacuum in the presence of desiccant and sodium hydroxide pellets. When dry, a few ml of water were added and the desiccation to dryness was repeated. The sample was then taken up in pH 2.2 citrate 0.2 M sodium ion automatic amino acid analyzer loading buffer. The dilution at this point depended on the objective of the experiment but typically was from five ml for one mg of starting protein to 25 ml for four mg.

2. Acid hydrolysis with methanesulfonic acid. The procedure is similar to hydrochloric acid hydrolysis with the following differences. One ml of the sample solution, containing up to five mg of protein is mixed with one ml of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (Pierce Chemical Co. product no. 2-5600 furnished in pre-scored one ml ampules under nitrogen) in a Carius (etc.) tube. The samples were degassed and sealed as described in (1). Heating typically was for 24 hours at 108° in an oil bath. The samples were then cooled, opened and neutralized (to pHydrion paper) with 4 N sodium hydroxide and 0.5 ml of saturated citric acid was added. Methanesulfonic acid is not very volatile and can not be removed by desiccation. The solutions were taken up in pH 2.2 loading buffer. Due to the salt concentration of the sample, about three ml, 1.3 M in sodium, it is desirable to dilute the sample as much as possible at this point to avoid disturbances in peak times on the analyzer. The effect of increased salt is to speed up most amino acids which not only complicates identification, but may cause loss of resolution.

B. Basic hydrolysis with sodium hydroxide. While many amino acids are destroyed to varying degrees, Met and MetSO usually can be recovered in good yields by this technique. Acid hydrolysis tends to reduce MetSO to Met. The method was adapted from Jori et al. (1969b).

One ml of the protein solution, usually containing five mg of protein, was mixed with one ml of 6.6 N sodium hydroxide, as carbonate free as possible, in a four ml screw cap Teflon vial (Misco Scientific, Cat. no. 0-9980). The samples were not degassed. The vial was capped and clamped in a c-clamp using coins, generally nickels, for shims. This was found to be necessary as the seal on the vials was otherwise undependable. The assembly was then placed in a oil bath, usually at 108-110° for 24 hours. After removal and cooling, 0.6-0.7 ml of 12 N hydrochloric acid was added and the solution was vacuum desiccated to dryness with sodium hydroxide and desiccant. A few ml of water was added and the desiccation was repeated. The samples were taken up in pH 2.2 citrate loading buffer and diluted to, usually, 25 ml to lower the salt concentration as much as possible.

Enzymatic Activity Assays

A. Activity analysis of lysozyme. Two different techniques were used. Both were based on the turbidometric measurement of the degree of clearing of suspensions of Micrococcus lysodeikticus. Initial work used a technique referred to Jori et al. (1969b) and decribed by Smoleis and Hartsell (1949). The basic procedure is to mix standard amounts of a M. lysodeikticus suspension with enzyme, wait a set time, 30 to 50 minutes, and determine the absorbance of the suspension. This technique is unsatisfactory. It gives usable results

only over a very small range of enzyme activity with considerable variation between duplicate determinations. The second technique follows the procedure of Gorin et al. (1971). In this, the initial rate of clearing of a suspension was determined. This method proved much more satisfactory and was used in later work.

1. Set time assay of Smoleis and Hartsell (1949). Assay buffer was prepared by dissolving 13.8 g (0.1 mole) of sodium dihydrogen phosphate monohydrate to one liter in distilled water and adjusting to pH 6.2 with dilute sodium hydroxide. Cellular suspensions were prepared by macerating approximately 40 mg of spray dried M. lysodeikticus cells (Miles Laboratory, control no. 79) in buffer and diluting to 100 ml. These concentrations have absorbances of 0.6-0.9. Standard lysozyme solutions were prepared by dissolving 20-30 mg of chicken egg white lysozyme (Sigma 3X recrystalized) in 10 ml of distilled water or the buffer and filtering through a 0.45 μm Millipore filter using a Swinny adapter. The solution was diluted to give 10-15 μg/ml of lysozyme, as noted. The concentration was checked by determining the absorbance at 280 nm and using the value of Canfield (Jori et al., 1969b) of $A_{280}^{1\%} = 26.4$. A standard curve was prepared by adding 3.0 ml of cells, and 0.1-1.0 ml of the standard lysozyme solution, making up the difference to 4.0 ml total with buffer. The absorbance would be determined at 540 nm in 30-50 min. This gave

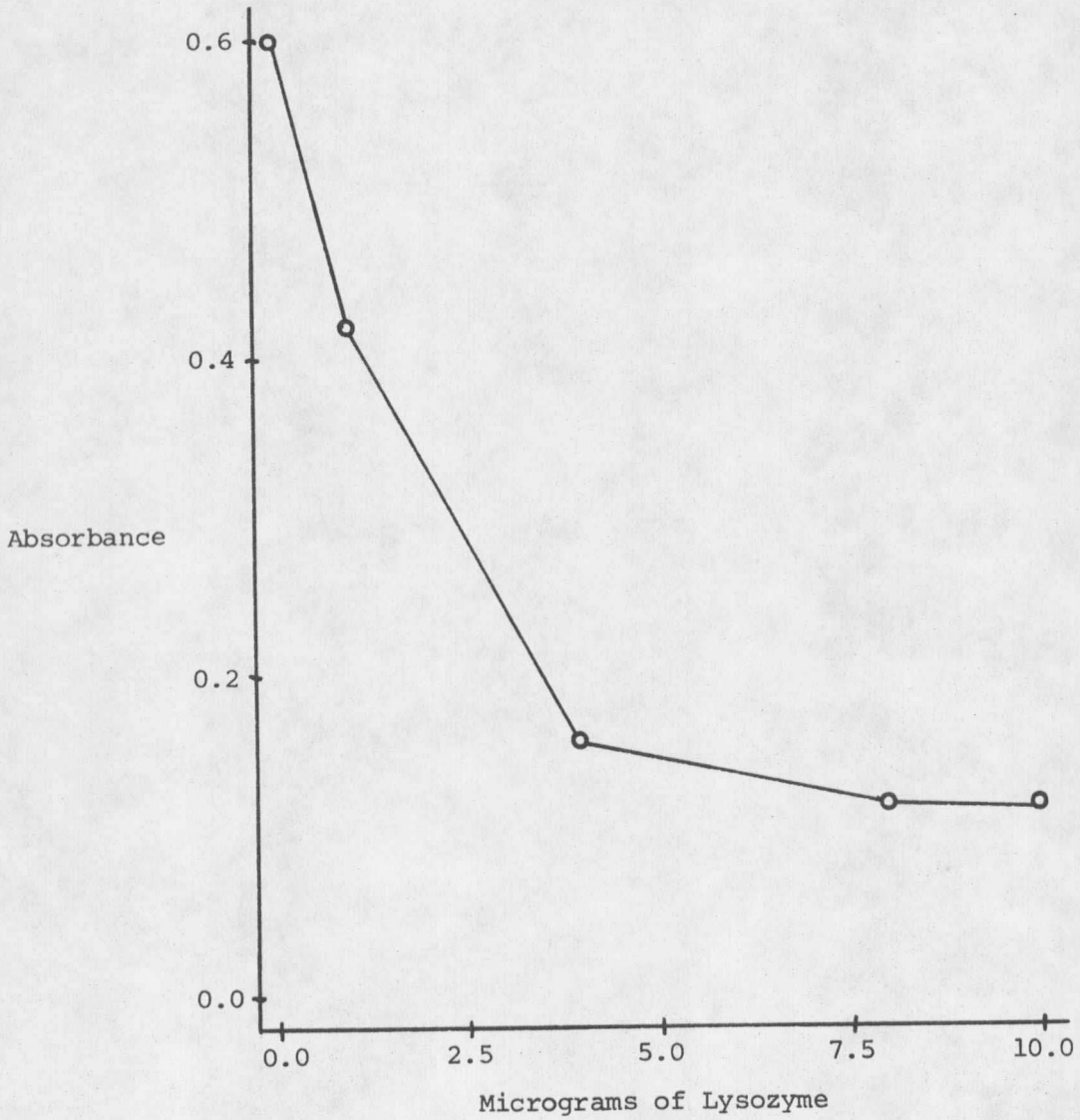


Figure 5. Standardization curve for the lysozyme assay of Smolelis and Hartsell.

values from approximately 1 µg/ml of lysozyme to 10 µg/ml. The unknown solutions were prepared, usually after gel filtration, as noted, by determining the concentration by the absorbance at 280 nm and using the perhaps unwarranted assumption that there would not be much Trp loss while any activity remained. The solutions were diluted to 10 µg/ml and several amounts of each, e.g., 0.1, 0.5 and 1.0 ml were added to 3.0 ml of cells and difference to 4.0 ml was made up with buffer. The same time would be used for all solutions, standard and unknown, during a determination. Absorbances were determined using either a Beckman DB using 1 cm cuvettes or Spectronic 20, using tubes. All temperatures were room temperatures and were maintained fairly constant during a determination. A graph of absorbance vs. µg of lysozyme standard shows a fairly steep linear relationship from 0 to 4 µg but above five µg the curve flattens out, permitting much less sensitivity. Results for unknown were reported in µg equivalents of lysozymes standard activity per 10 µg of lysozyme. Figure 5 shows a typical standardization curve.

2. Initial rate assay of Gorin et al. (1971). The buffer, LZ, was prepared by dissolving 10.37 g of sodium dihydrogen phosphate monohydrate, 3.12 g of disodium hydrogen phosphate and 0.372 g of disodium dihydrogen ethylenedinitrilotetraacetic acid dihydrate (EDTA) and diluting to one liter. This has a pH of 6.2 and is 0.1 M in

phosphate and 10^{-3} M in EDTA. The substrate was prepared by macerating 20-25 mg of M. lysodeikticus cells in 10-15 ml of the buffer, using a fitted Teflon piston tissue homogenizer, and diluting to 100 ml. The standard and unknown lysozyme solutions, in buffer, were prepared as in the preceding section but to concentrations of 10 to 100 µg/ml. Unknowns were usually set to 100 µg/ml. In some cases, as noted, protein concentrations were determined by the Lowry method.

The assays were run by mixing 3.0 ml of the cells suspension with 0.10 ml of the enzyme solution, quickly mixing and transferring to a 1 cm cuvette in a Beckman DU-2. The absorbance reading at 570 nm was started within 40 secs. Using a stop watch, the time necessary for the absorbance to decrease 0.05 was measured. Depending on the cell preparation and enzyme concentration, initial readings were from about 0.65 down to 0.50 and times varied from 10 secs. to over five minutes. The reciprocal of the time was graphed vs. µg of standard lysozyme added. This gave a linear range from 2 µg to 20 µg. The non linear decrease in time at values less than 2 µg seemed due to warming of the solution in the DU-2 because of the length of the time involved. All other temperatures were room temperature. Precision of this method, after practice, seems good. Approximate precision was 5%. Figure 6 shows a typical standardization curve obtained early in this work. With experience, very little data scatter can be achieved.

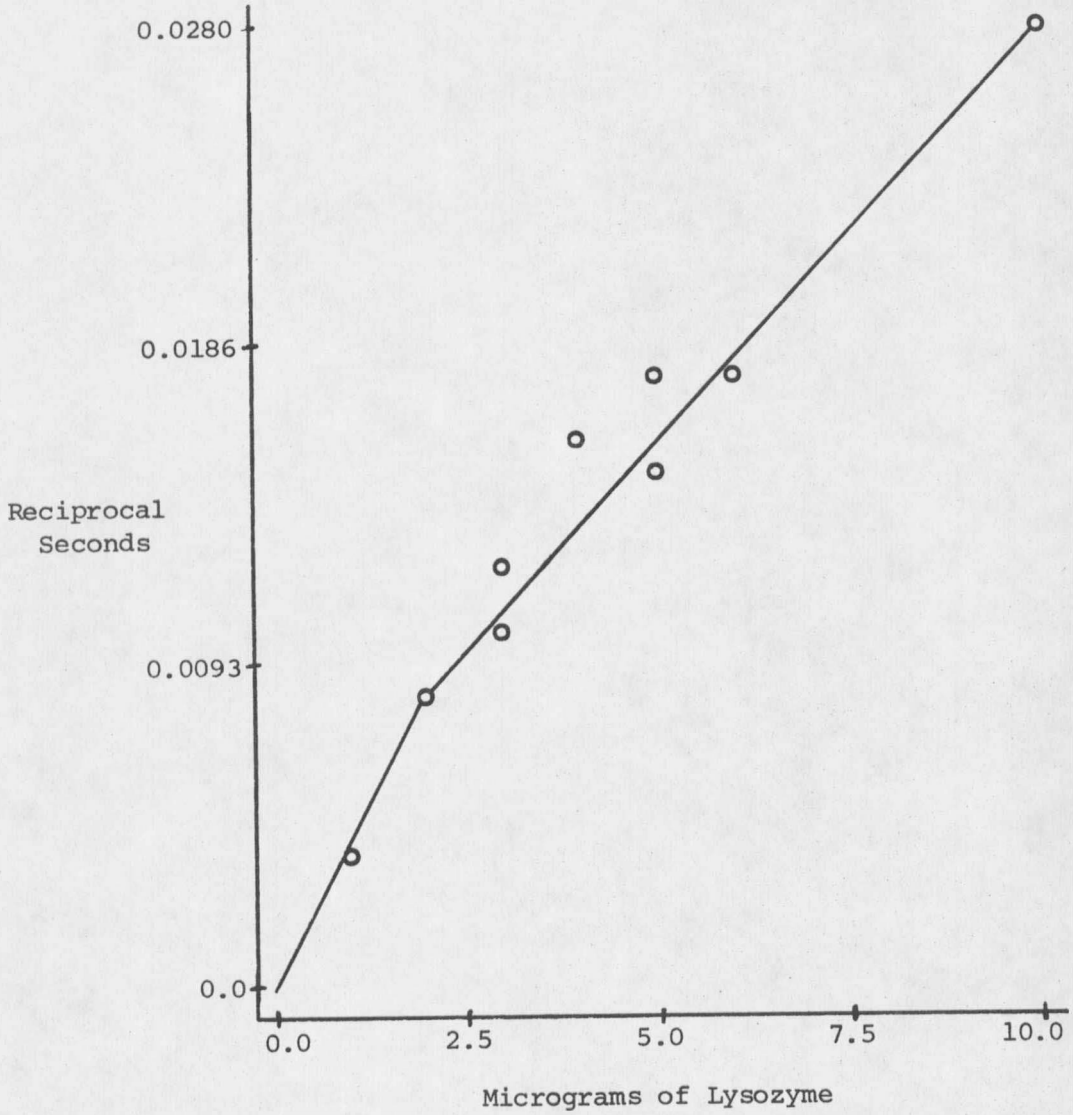


Figure 6. Standardization curve for the lysozyme assay of Gorin, Wang and Papapavlou.

B. Kunitz ribonuclease assay (1946). The method is based on the slight, 10-15%, decrease in absorbance at 300 nm of soluble ribonucleic acid in pH 5 buffer upon hydrolysis by ribonuclease. The initial rate of this decrease is proportional to the concentration of enzyme. The method requires a very stable spectrophotometer capable of being read accurately to ±0.002 at an absorbance of 0.5-0.6. Twenty-six mg of yeast RNA (Sigma, type XI, no. R6750, lot 98B-8050) was dissolved in 40 ml of buffer RN (0.1 M pH 5.0 acetate). The absorbance at 300 nm was 0.595. Two ml of this were mixed rapidly with 0.100 ml of the enzyme solution and its absorbance at 300 nm was followed. The activity assay is useful over final concentrations of enzyme in the 2.1 ml of from about 0.2 to 2.0 µg/ml. As soon after mixing as possible, usually 20 sec., the absorbance at 300 nm was determined and then at one minute and integral minutes for up to 30 minutes depending on the rate of decrease. The absorbances were plotted against time and the initial slope of the decrease was determined. The slopes were plotted against concentration of enzyme.

Gel Filtration on Sephadex G-25

When it was necessary to separate a small molecular weight substance, such as a dye, non-volatile buffer or salt from a protein, gel permeation chromatography was used. The general technique was as follows with specific exceptions noted in individual experiments. A 50 ml

buret with Teflon stopcock was used for the column. Approximately eight g of medium Sephadex (Pharmacia lot 390) was soaked overnight in the eluent, either water or 5% acetic acid, using several changes, and poured into the column. This gives a 25-27 ml volume bed. The column was plugged at the bottom with short fiber fiberglass wool. One to two hundred ml of the eluent was run through the column to wash it and the level of liquid was dropped just to the surface of the packing. The sample was put on in about one ml of liquid and drained down to the surface of the packing. One ml of the eluent was carefully added and allowed to drain. Several fractions were collected. Blue dextran comes through at from 10-15 ml with a peak at 12 ml. Protein fractions were usually collected at from 8-18 ml. Hematoporphyrin was eluted around 50 ml. The column was then washed with a 100 ml of the eluent to clean it for reuse. Sometimes it was necessary to use 10% acetic acid to remove hematoporphyrin precipitates.

Inactivation of Lysozyme in Solution by Oxidation with Microwave Discharge Generated Singlet Oxygen

The microwave discharge reactor was set up as described. The Evanson Cavity and 10% oxygen in helium were used. The settings were 30 torr pressure and 75% power.

Fifty ml of a 10 µg/ml water solution of lysozyme were oxidized. This concentration is appropriate to use directly in the enzyme

activity assay of Smolelis and Hartsell (1949) so no further preparation is necessary. Five ml samples were withdrawn at 0, 0.5, 1.0 and 2.0 hrs. A Spectronic-20 was used in the assay. Standard tubes used 0, 0.1, 0.4 and 1.0 ml of the starting solution. Aliquot of 0.2 and 1.0 ml of the 0.5 and 1.0 hour and 0.4 and 1.0 ml of the 2.0 hour samples were used in the assay.

A control of the same concentration of lysozyme was run at the same conditions of pressure and time except that the microwave power was not turned on. Control samples were taken at 1.0 and 2.0 hours.

Oxidation of Amino Acids by Microwave Discharge Generated Singlet Oxygen

A. Photooxidation of amino acids. For standards of comparison, the photooxidation products of dye sensitized photolabile amino acids were prepared. The amino acids and peptides were Met, N-acetyltryptophan (NAT), N-glycyltryptophan (NAG), N-benzoylhistidine (NBH), Trp, His and as a control, alanine (Ala). Rose bengal and methylene blue were used as sensitizers. In a typical reaction, 0.1 g of the substrate amino acid or peptide was dissolved in 50 ml of 0.04 M pH 9 potassium phosphate buffer. The dye concentrations were 50 mg/50 ml for rose bengal and 25 mg/50 ml for methylene blue. During photooxidation, the solutions were aerated and stirred by a slow stream of filtered compressed air. Illumination was provided by either a 150 watt G.E. reflector spot-lamp or a 600 watt Sylvania type

FCB tungsten-halogen projector lamp run at 80% power (approximately 95 volts) by means of a variable autotransformer (Staco model 3 PN 1010). The solutions were illuminated at a distance of 10 cm through the bottom of a 125 ml Erlenmeyer flask in an 800 ml beaker which served as a water bath. Cooling was provided by immersing a coil of copper tubing connected to a water tap in the bath. Temperatures were maintained at 10-20°C. Illumination times of up to 250 minutes were used. Samples were withdrawn at various times during the illuminations for analysis by thin layer chromatography. If it was considered necessary on a particular sample, the dyes were removed by running the sample through an appropriate Sephadex (Pharmacia Fine Chemicals, Inc.) ion exchange resin. Two ml's of the sample solution were run through three cm beds of the resin, preequilibrated in the pH 9 buffer, in six mm disposable pipettes. Rose bengal was removed with A-25 course DEAE Sephadex and methylene blue was removed by C-50 CM-Sephadex. The sources of the amino acids and peptides are as below:

Material	Supplier	Supplier Code	Lot Number
Glycyltryptophan	Sigma Chemical	G-3377	101c-3020
N-acetyl-Dl-tryptophan	Sigma Chemical	A-6251	120c-0840
L-Histidine	Sigma Chemical		86B-0210
DL-Methionine	Mann Research Laboratories	6952	S-1852
L-Tryptophan	Nutritional Biochemicals Corp.	1-1315	not listed
DL-Alanine	Matheson Co.	2223	not listed
N-Benzoylhistidine	prepared as described below		

1. Preparation of N-Benzoylhistidine using the conditions of E. Fischer (1899). His (2.8 g 0.018 mole Sigma lot 86B-0210) was dissolved in 100 ml of doubly distilled water with 13 g of sodium bicarbonate. Benzoyl chloride (7.7 g, 0.055 mole) was added in small portions with stirring. The solution was extracted with 50 ml of diethyl ether to remove unreacted benzoyl chloride, acidified and allowed to stand. The only product observed was an intractable oil. No further work up was done.

2a. Using the conditions of Stieger (1944). Histidine hydrochloride (4.2 g, 0.02 mole, Baker, code N342, lot 2-0106) was dissolved in 80 ml of 1 N sodium hydroxide (0.08 mole) and cooled in an ice bath. Benzoyl chloride (2.9 g, 0.0206 mole) was added in small portions over half an hour with stirring. After all the benzoyl chloride had been added, the solution was allowed to warm to room temperature and sat for an additional half hour with continuous magnetic stirring. The solution was extracted with 80 ml of diethyl ether and then acidified to pH 3 with hydrochloric acid. A white precipitate formed which was filtered off. The precipitate was extracted with small portions of petroleum ether and recrystallized from hot water. Air dried on a Buchner funnel, the melting point was 112-120°C. This material seemed to be benzoic acid, mp 122°. The pH of the mother liquor from the crystallization was adjusted to 4-5, to

approximate the estimated isoelectric point of N-Benzoylhistidine, and the solution was boiled down to about 25 ml and allowed to stand overnight. A second crop of white crystals was filtered off and air dried on the filter. Yield 0.58 g, mp 233-245° with decomposition. The crystals were washed with 60 ml of hot acetone and 40 ml of hot benzene by boiling. The insoluble crystals were filtered off and air dried. The melting point did not change. Reported melting points of NBH, 249° and 230° by Shriner et al. (1956) (original source unknown). Thin layer chromatograms of this material were unreactive with ninhydrin, reactive with the Pauly reagent and quenched fluorescence, which is consistent with NBH (Tomita et al., 1969).

b. As in (a.) with the following exceptions. The His hydrochloride was dissolved in 40 ml of 1 N sodium hydroxide (0.02 moles of His•HCl and 0.04 moles of NaOH) and 0.02 mole of benzoyl chloride was added with 20 ml (0.02 mole) of 1 N sodium hydroxide in small portions over half an hour. The solution was allowed to set for half an hour with stirring and then extracted with two 40-50 ml portions of ethyl ether. The pH was adjusted to about 40 and the solution was placed in a refrigerator for three hours. The crop of white, long needle-like crystals were filtered off and washed twice with 10 ml portions of acetone. Yield: 2.67 g (51%) mp 244-246° with decomposition. The crystals decrepitate at 110-130°.

B. Oxidation of amino acids in solution by microwave generated singlet oxygen. The same amino acids that were photooxidized in the preceding section were oxidized using the same pH 9 potassium phosphate buffer and amino acid or peptide concentrations. This concentration was usually 0.1 g/50 ml. Reactions were run in the microwave reactor as described. Settings were at 15-20 torr and 80% power using 10% oxygen in helium. Fifty ml of solution was placed in the 3 × 20 cm flask. Reactions were run for 150 to 210 minutes. After 3.5 hours, it was usually necessary to disassemble the equipment for cleaning as mercuric oxide and ice deposits had built up to the point that operation was difficult. The gas stream was bubbled into substrate solutions using a course glass frit. These conditions gave very vigorous bubbling. At the end of a reaction run, the substrate solutions were stored in a refrigerator and analyzed by thin layer chromatography for extent of oxidation and the nature of the products.

Thin Layer Chromatography of Amino Acids

Thin layer chromatography (TLC) was used to analyze some of the results of sensitized photooxidations and microwave generated singlet oxygen oxidations of amino acids.

A. Thin layer materials. A number of different thin layer materials were used. Most of the work was done using various solvents with Eastman silica gel sheets with fluorescent indicator. Initially,

this was EK 6060, then after a product code change, EK 13191. This last is also prescribed but apparently chemically equivalent to the EK 6060. A few experiments used Bakerflex 1B silica gel sheets. Some work was done using Macherey-Nagel MN-Polygram Ionex-25 SA-Na sulfonate ion-exchange resin TLC sheets. This material offers a TLC approach which is chemically analogous to the column chromatography of the automatic amino acid analyzer. Buffers of the same general types as used in analyzers are used as eluents. Some work also used MN-Polygram Cel 300 microcrystalline cellulose sheets. In one series of determinations using phenol based eluent, freshly prepared glass TLC silica gel-H plates were used as the EK 6060 is decomposed by phenol solutions.

These plates were prepared by rapidly grinding 10 g of silica gel-H (Merck-Darmstadt prepared according to Stahl) with 25 ml of water in a mortar until smooth. This was then poured on very clean, dry 20 × 20 cm glass plates and smoothed down with a one cm diameter glass rod. The sides of the plates were shimmed with two layers of black plastic electricians tape (U.P.A. International) to give layers 300-400 μm thick. The plates were air dried overnight before use, but not heat activated.

B. Containers. Chromatographic developments were run in two different types of jars, depending on the size of the sheet or plate.

The 20 cm long sheets or plates, of whatever width, were developed in 12 × 24 × 23 cm rectilinear "battery" jars covered with glass plates. Small strips of sheet, and in one case carboxymethyl cellulose layers on microscope slides, were run in beakers covered with watch glasses. The most commonly used were tall form 180 or 200 ml beakers about 11.5 cm tall. Both the jars and the beakers were lined, to the extent of 50-80% of the circumference, with sheets of filter paper which were saturated with eluent to permit rapid equilibration of the vapor phase. During early work, the spotted sheets or plates were allowed to stand in the jars, before dipping in the eluent, for periods of from two to 12 hours, to pre-equilibrate the layers with the eluent vapor. This practice proved to be undesirable due to spot spreading, especially laterally. Resolution seemed as good or better and detection was more sensitive with immediately eluting the plates when they were placed in the jars. There was some difficulty with uneven fronts.

C. Eluents. The following solvents used with silica gel:

1. Butanol-Acetic acid-Water(4:1:1 by vol.)
2. t-Amyl alcohol-Pyridine-Water(35:35:30 by vol.)
3. Methanol-Chloroform-17% ammonium hydroxide(40:40:20 by vol.)
4. Phenol-Water(75:25 by wt. on fresh Silica Gel-H)

5. 96% Ethanol-34% ammonium hydroxide(70:30 by vol.)

6. Propanol-34% ammonium hydroxide(70:30 by vol.)

7. pH 4.25, 0.067 M citrate buffer on Ionex-25-SA

All of these solvents are described by Brenner et al. (1965) in "Thin Layer Chromatography" edited by Stahl. They recommend solvents 1, 3 and 4 for the best general resolution of amino acids and the experience of this work tends to confirm this. Solvent "one" proved to be the most generally useful ("three" was unstable due to volatility and phenol presented handling problems besides requiring glass plates). Solvent "one" will occasionally be referred to by the acronym "BAW".

The recipe for the solvents used with the MN-Ionex 25-SA-Na were obtained from the package insert which referred to work done by Devenyi. A pre-equilibration buffer was prepared by diluting 1:10 a solution composed of 14.1 g of citric acid monohydrate, 12.3 ml of concentrated hydrochloric acid and 8.0 g of sodium hydroxide in one liter of water. The diluted solution has a pH of about 3.5 and a sodium ion concentration of 0.08 M. The eluting buffer was a pH 4.25 solution of 14.1 g citric acid monohydrate, 8.0 g of sodium hydroxide, 8.4 ml of concentrated hydrochloric acid, and 35.0 g of sodium chloride in one liter. The sodium ion is 0.8 M and the citrate is 0.065 M. The sheet is chromatographed with the equilibrating buffer for three hours (takes about an hour to go to the end) at room

temperature. The sheet is then air dried at room temperature. The sample is spotted on in a thin line and allowed to dry. The sheet is then chromatographed with the eluting buffer for two hours. Maintaining the chamber at 50°C improves resolution. The sheet is oven dried for 5-10 minutes at 100-110° before visualization.

D. Visualization

1. Ninhydrin: The amino acids and peptides which had a free α-amino groups were visualized with ninhydrin (Ninspray). The free amino acids give the purple (rosy in presence of residual phenol) color while the peptides (which do not have an amino alpha to a carboxylate) give yellow with less sensitivity. The sheets were thoroughly dried after eluting then sprayed and heated for a few minutes, either in a 100° oven or by a hair drier-heat gun. The exception to the thorough drying was with BAW, where it was found that a slight residue of acetic acid improved development. The ammonia based eluents especially had to be removed for good results.

2. Ehrlich's reaction for tryptophan and its derivatives: This is a general color reaction of acidified 4-dimethyl-amino-benzaldehyde with indole derivatives. It produces color with a number of other structures. Of special interest here is the color with kynurenine (Kyn) and its N′-formyl derivative. The reagent was used in two variations. In early work a spray solution of one g of

4-dimethylaminobenzaldehyde in 50 ml of ethanol plus 50 ml of concentrated hydrochloric acid was used. This was hard on the sheets causing them to peel if care was not taken. Also, if the 4-dimethylaminobenzaldehyde is applied first, before acidifying, the yellow Kyn spots appear more strongly. They fade somewhat upon acidification when the purple Trp (indole) color develops.

To see the initial Kyn reaction, a 0.5% solution of the aldehyde in 2-propanol (Merck-Darmstadt) was sprayed on the chromatograms and allowed to dry. This will give the yellow Kyn colors. Then the chromatogram was placed in a jar of hydrogen chloride vapors for from three to ten minutes to develop the purple color. If left in too long, the Kyn color tended to fade but it usually reintensified after standing.

3. Pauly's reagent for histidine imidazole: This is a diazotization reaction of the imidazole nucleus (Brenner et al., 1965, p. 488). Two solutions are sprayed: Solution "one" is prepared by slowly adding 25 ml of freshly prepared 5% sodium nitrite at 0° to five ml of 0.9% sulfanilic acid in 1.1 M hydrochloric acid. Solution "two" is 5% sodium carbonate. Solution "one" is sprayed and allowed to dry slightly. While still moist, the chromatogram is sprayed with solution "two". The intense orange color produced is quite stable.

E. Application of analytes. Analytes were applied to the chromatograms by means of a Hamilton 10 µl syringe equipped with an incremental dispenser (also Hamilton) which permitted increments of approximately 0.2 µl. Spots were usually placed 2.0-2.5 cm from the bottom edge. They were one cm apart on unscored sheets or plates. The spot size was usually about two mm in diameter except for the Ionex-25 where a 1 × 5 mm line was spotted. Amino acids and peptides were spotted in water solution, except for cystine and tyrosine which were applied in 0.1 N hydrochloric acid as they were not easily soluble in water. Solutions of one µg/ml to 10 µg/ml were used. Usually an amount equivalent to 10 µg of analyte was spotted with drying by a heat gun between 0.2 µl applications.

Effect of Singlet Oxygen Quenchers and Enhancers on Hematoporphyrin Sensitized Photooxidation of Lysozyme

A. Experimental conditions were adapted from Nilsson and Kerns (1973).

1. Lysozyme (Sigma lot 93C-8000) was prepared by dissolving 23 mg 1.60×10^{-6} mole in 10 ml of distilled water and filtering (Millipore HAWP 0.45 µm). The solution was diluted to 12 ml (approximately 1.33×10^{-7} mole/ml) and equally divided. Hematoporphyrin was prepared by dissolving 60 mg (8.9×10^{-5} mole) of the hydrochloride in five ml of ethanol (0.018 M). Sodium azide was prepared by dissolving 653 mg (10^{-3} mole) in 100 ml of buffer. Six ml of the lysozyme

preparation (8.0×10^{-7} mole), 0.7 ml of either the azide or the LZ buffer, for a control, and, in the dark, 0.1 ml (1.8×10^{-6} mole) of the hematoporphyrin. The solutions were 257 μM in dye and 114 μM in lysozyme. The solutions were placed in a 50 ml volumetric flask in an ice bath and illuminated through the bottom by a 150 watt spot lamp. Samples of 0.7-0.8 ml were withdrawn at 0, 1, 2, 4, 8, 16, 32 and 48 minutes. The solutions were gel filtered, lypholyzed, and taken up in 3-4 ml of LZ buffer. Note that the molar dye:lysozyme ratio was approximately 2.25.

2. The protein concentration of these samples was determined on 0.5 ml aliquots by the Lowry Method, standardized against solutions containing 50-250 μg/ml of lysozyme prepared from the stock preparation in (1). The solutions were each diluted to 100 μg/ml using buffer LZ and the absorbance at 280 nm was determined.

3. The enzyme activities were determined by the method of Gorin. Standards of 10, 20, 30, 40, 50, 60, 100 and 200 μg/ml were used. All points except 100 μg/ml were in duplicate. Four determinations were made at 100 μg. A single determination apiece of the unknowns was made at each time points.

B. Essentially the same as (A) using azide and 1,4-diazabicyclo-2.2.2.octane (DABCO) at one tenth the concentration as

inhibitors and looking at the amino acid hydrolysis products of the final oxidized material. A pH 6.2 0.1 M phosphate buffer was prepared by dissolving 10.37 g of sodium dihydrogen phosphate monohydrate and 3.12 g of disodium hydrogen phosphate to one liter. Lysozyme, 150 mg (Sigma lot 93C-8000), was dissolved in 100 ml of the buffer. A 1:10 dilution had A_{280} = 0.368, equivalent to 96 µM in the stock solution. One hundred mg of hematoporphyrin dihydrochloride (1.5×10^{-4} mole) (Fluka lot 701459) was dissolved in 50 ml of 1.2×10^{-2} M sodium hydroxide (6×10^{-4} mole) to give 300 µM dye. The inhibitors were prepared in 10^{-2} M concentration by dissolving 65 mg of sodium azide and 112 mg g of DABCO in 100 ml apiece of the 6.2 buffer. An irradiation mixture consisted of 24 ml of the lysozyme solution (2.3 µmoles), 2.5 ml of the dye (7.5 µmoles) plus 3.0 ml of either buffer (control) or one of the inhibitors. The solutions were 76 µM in lysozyme and 250 µM in dye. The solutions were irradiated in a 50 ml volumetric flask in an ice bath using the mirror-750 watt projector combination. An air stream was directed over the surface of the solution, which was occasionally stirred. The pH of all solutions were 6.2. Samples of 0.60 ml were withdrawn at 0, 1, 2, 4, 8, 16, 32, 64 and 128 minutes. The samples were mixed with one ml of 10^{-2} M sodium azide and diluted to 10 ml with LZ buffer. Because of the extreme dilution of the dye in an assay, the dye was not removed before analyzing the enzymatic activity by the Gorin method. Twenty-five mg of M. lysodeikticus

cells were suspended in 100 ml of LZ buffer. Amino acid analyzer codes were: control, acid hydrolysis 788-74, base 789-74; azide, acid 790-79, base 791-74.

C. This experiment was similar to (B), the main difference being in the buffer systems used. The object of the experiment was to compare the relative rates of hematoporphyrin sensitized photo-oxidation in deuterium oxide, water and azide-water as in Nilsson and Kearns (1973). Deuterophosphoric acid was unavailable, so an acetate buffer was prepared by the reaction of acetic anhydride and deuterium oxide plus sodium acetate. Controls were prepared the same way with water.

1. Water systems: The buffer was prepared by dissolving 61.5 mg (7.5×10^{-4} mole) of sodium acetate and 38.2 mg (3.7×10^{-4} mole) of acetic anhydride in water, made to 15 ml, and allowing the solution to stand an hour. This gives a 0.1 M buffer of pH 4.3. Lysozyme was prepared by dissolving 33 mg in eight ml of the buffer, filtering (0.45 μm Millipore) and diluting to 18 ml with water. The absorbance of a 1:10 dilution of this at 280 nm was 0.423, equivalent to 1.6 mg/ml (1.1×10^{-7} mole/ml) in the stock dilution. The hematoporphyrin and azide preparations from (A) were used. Photooxidation mixtures consisted of six ml of the lysozyme (6.6×10^{-7} mole), 0.1 ml (1.8×10^{-6} mole) of the dye and 0.7 ml of either azide or water.

The mixtures were illuminated as in (A). Approximately 0.5 ml samples were taken at 0, 1, 2, 4, 8, 16, 32, 64 and 128 min. The pHs of the final solutions were checked in each case, being 4.3 for the water control and 4.7 for the azide. The samples were gel filtered, lypholyzed diluted to 100 µg/ml (6.2 buffer), based on 280 nm absorbance, and their enzymatic activity was determined. The protein concentrations were later checked by means of the Lowry Method.

2. Deuterium system: The buffer was prepared by adding 42.7 mg (5.2×10^{-4} mole) of sodium acetate and 26.4 (2.6×10^{-4} mole) to 11.6 g (10.5 ml) of deuterium oxide (99.87 mole % D_2O, Bio-Rad control number S-11981) and allowing to stand one hour. The pH was 4.75 with 0.1 M total acetate. Lysozyme was prepared by dissolving 23 mg in the buffer. The absorbance of a 1:10 dilution of this in LZ buffer was 0.466, equivalent to 123 µM in the stock. The stock was diluted 1:1.2 (8.8 ml to 10.5) with deuterium oxide to give the same concentrations (103 µM) as in (1). The photooxidation mixture was 6.7 ml of the lysozyme (6.9×10^{-7} mole) and 0.1 ml (1.8×10^{-6} mole) of dye. The rest of the experiment was as in (1). Note that the dye/lysozyme molar ratios in these experiments were approximately 2.6:1.

3. Enzyme assay (Gorin Method): M. lysodeikticus cells were prepared by suspending 50 mg in 200 ml of buffer LZ. Lysozyme standards were prepared by dissolving 30 mg in 25 ml of LZ and

filtering. A 1:10 (5:50) dilution of this had an absorbance of 0.300 at 280 nm. This dilution was equivalent to 113 µg/ml. This dilution was diluted 1:10, 2:10, 4:10, 5:10, 6:10 and 8:10 in LZ buffer as in (A). All standards were determined three times and all unknowns once, except for 0, 4 and 16 minute points of the water only control which were done in duplicate.

The solutions remaining at the end of photooxidation with the control and azide inhibited reaction were evaporated in a rotary film evaporator to four and two ml, respectively. The solutions were acidified with hydrochloric acid until the dye dissolved. The protein was precipitated and the dye removed by mixing the solutions with cold acidified acetone (1% hydrochloric acid) at the rate of two ml of solution to 12 of acetone. The tubes were centrifuged and the supernate decanted. The protein was redissolved in two ml of water and reprecipitated twice. The final pellet (∿0.2 ml) was dried by a gentle air stream and dissolved in two ml of water. The solutions at this point was yellow-brown suggesting melanines or kynurenines. One ml apiece of each of the solutions was hydrolyzed by methanesulfonic acid and sodium hydroxide hydrolyses. The amino acid analyzer run codes were: control acid 788-74, control base 789-74, azide acid 790-74 and azide base 791-74. The basic hydrolyses were run only on the long (acidic and neutral residues) column.

D. Essentially the same as (C) with the following differences. The effects of azide and deuterium oxide were investigated in both pH 4.7 0.1 M acetate and pH 6.2 0.1 M phosphate systems. A simplified buffer preparation was used as it was calculated that even in the presence of 0.1 M phosphate or acetate prepared from H^+ containing materials and 100 μM lysozyme that the concentration of hydrogen would be less than 1 M vs. ca. 110 M for deuterium, which should shorten the lifetime of singlet oxygen less than 10%. The azide concentration in the inhibition reactions was 0.2 M instead of 0.1 M used in other experiments. The 750 watt projector-mirror illumination system was used to illuminate the solutions through the bottom in 50 ml volumetric flasks in a clear ice bath.

The concentrations of lysozyme in these determinations were all 100 μM. The dye concentration was nearly exactly twice that. Photooxidation solutions were prepared according to Table 1.

Just before the start of light exposure, the solutions were mixed with 0.10 ml of 0.041 M hematoporphyrin (137.8 mg, 2.05×10^{-4} moles, of the dihydrochloride in 5.0 ml) in ethanol. The solutions were sampled at 0, 1, 2, 4, 8, 16, 32 and 64 minutes. The deuterium solutions were additionally sampled at 0.5 and three minutes. Samples of 1.0 ml were taken and added to 9.0 ml of LZ buffer and stored in the dark in a refrigerator. Since using these solutions directly in the Gorin activity assay dilutes them 1:30, giving an insignificant