



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
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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.

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OXYGEN ON PROTEINS AND AMINO ACIDS

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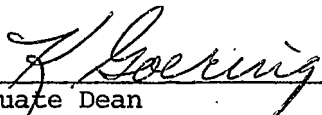
in

Chemistry

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Bozeman, Montana

September, 1974

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Samuel Rogers for his patience, guidance and assistance during the course of this research project. I would also like to thank the following people: Dr. Gordon Julian for guidance and encouragement, Dr. Kenneth Hapner for training and assistance in the operation of the amino acid analyzer, Dr. Ray Woodruff for guidance in the use of radiofrequency discharges, and Dr. Paul Jennings for assistance with mass spectra and their interpretation. The editorial efforts of members of my thesis committee, Drs. Arnold Craig and John Robbins, are gratefully acknowledged.

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.

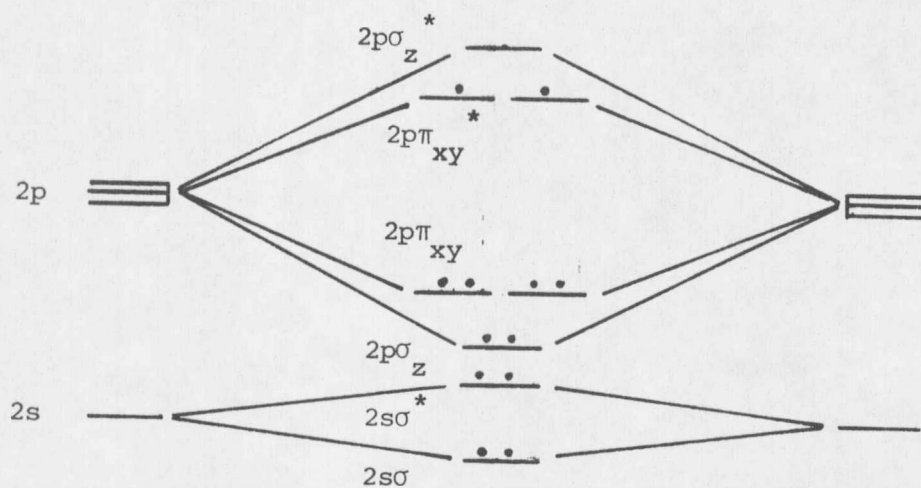
A number of different mechanisms have been proposed to account for photodynamic action. Until fairly recently, the most generally accepted views that the dyes absorbed light to give an excited dye electronic state. This either reacts with substrate to abstract a hydrogen atom or electron, thereby oxidizing 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

		Energy above Ground State
$1\Sigma_g^+$		37 Kcal
$1\Delta_g$		22
$3\Sigma_g^-$		0

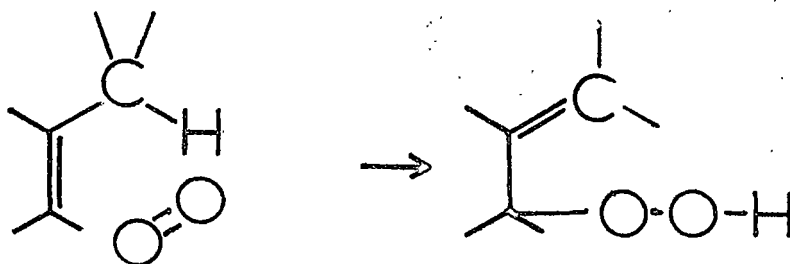
b. π_{xy}^* Orbitals showing Ground and Excited States

Figure 1. Molecular electronic state of oxygen.

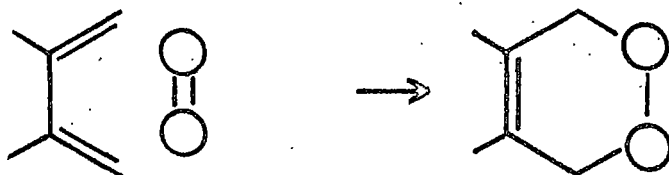
sensitizer were absorbed on separate grains of silica gel, allowing only for transfer of excited oxygen between them.

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

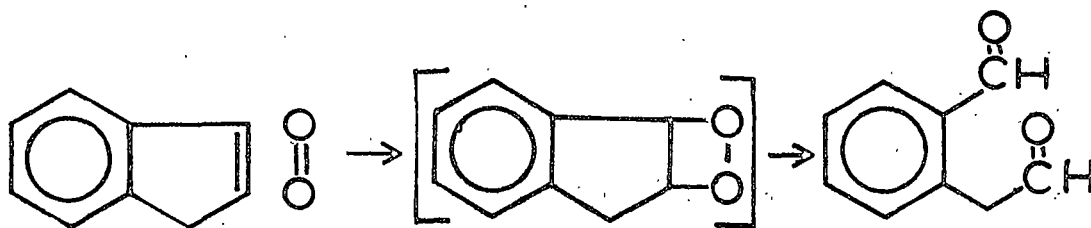
It is possible to generate singlet oxygen, especially the $^1\Delta_g$ state, by a number of methods which are independent of dye and light. When oxygen so generated is allowed to react with olefins which give distinctive ratios of products with dye sensitized photooxidation, the same products in approximately 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 polycyclic 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 μ seconds vs. 2 μ seconds) 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 transition 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 chemiluminescence 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 in 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, Cys₂). 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

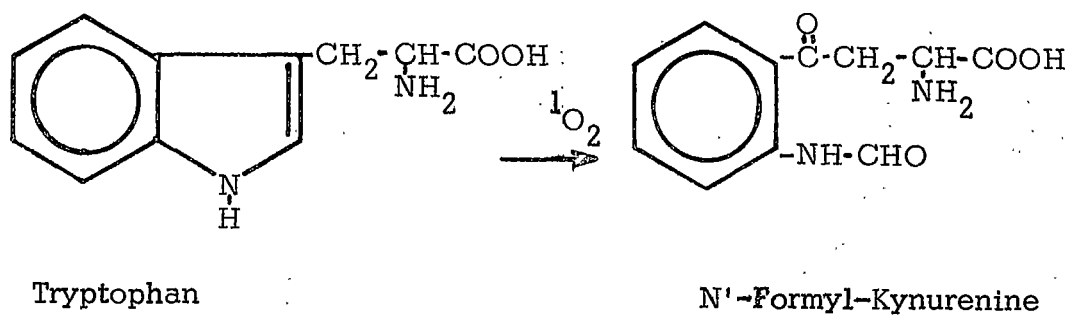
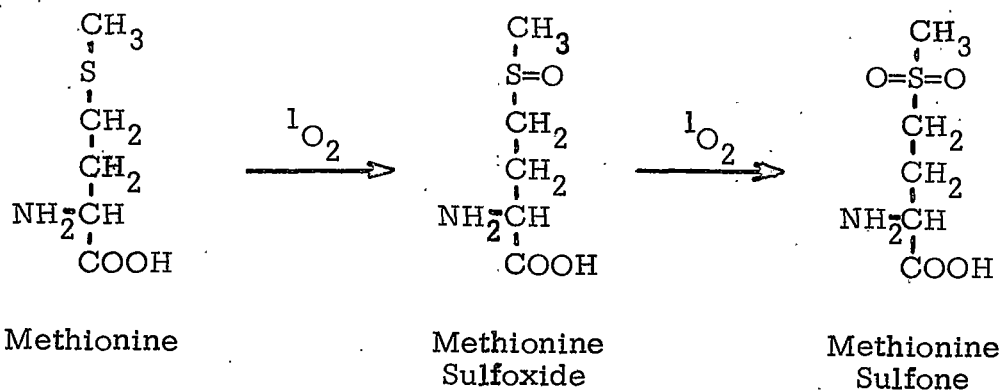


Figure 3. Products of photooxidation of methionine and tryptophan.

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 Cys₂, 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 Cys₂ 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 Trp paralleled that of the photooxidized reaction. They did not identify any amino acid products.

Effects that are so far not explained with regard to the photooxidation 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 $V(\text{Trp})/V(\text{His})$ varies from 0.9 for eosine Y to 0.25 for thionine. Thionine is noted in one study with dimethylantracene, 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, Cys₂, 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, Cys₂, 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 absorption 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 photo-oxidation 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 sulfonoxide.

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 $\frac{1}{8}$ inch O.D. glass and an 18 foot column of $\frac{1}{8}$ 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 valves. A "good" loading of a particular amino acid is 0.02-0.3 μ mole/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 μ mole, 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

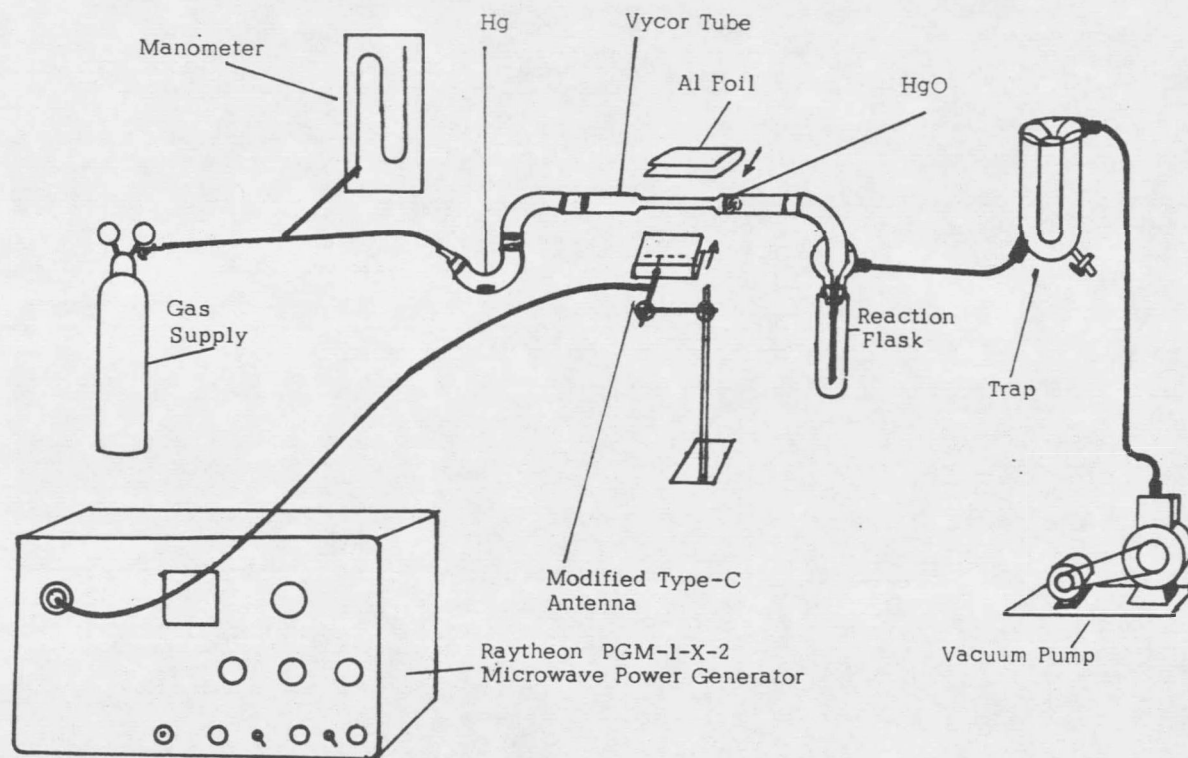


Figure 4. Microwave discharge singlet oxygen generator.

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 x 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 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-2-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 pHDrion 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 dependable. 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 described 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 recrystallized) in 10 ml of distilled water or the buffer and filtering through a 0.45 μ m Millipore filter using a Swinny adapter. The solution was diluted to give 10-15 μ g/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

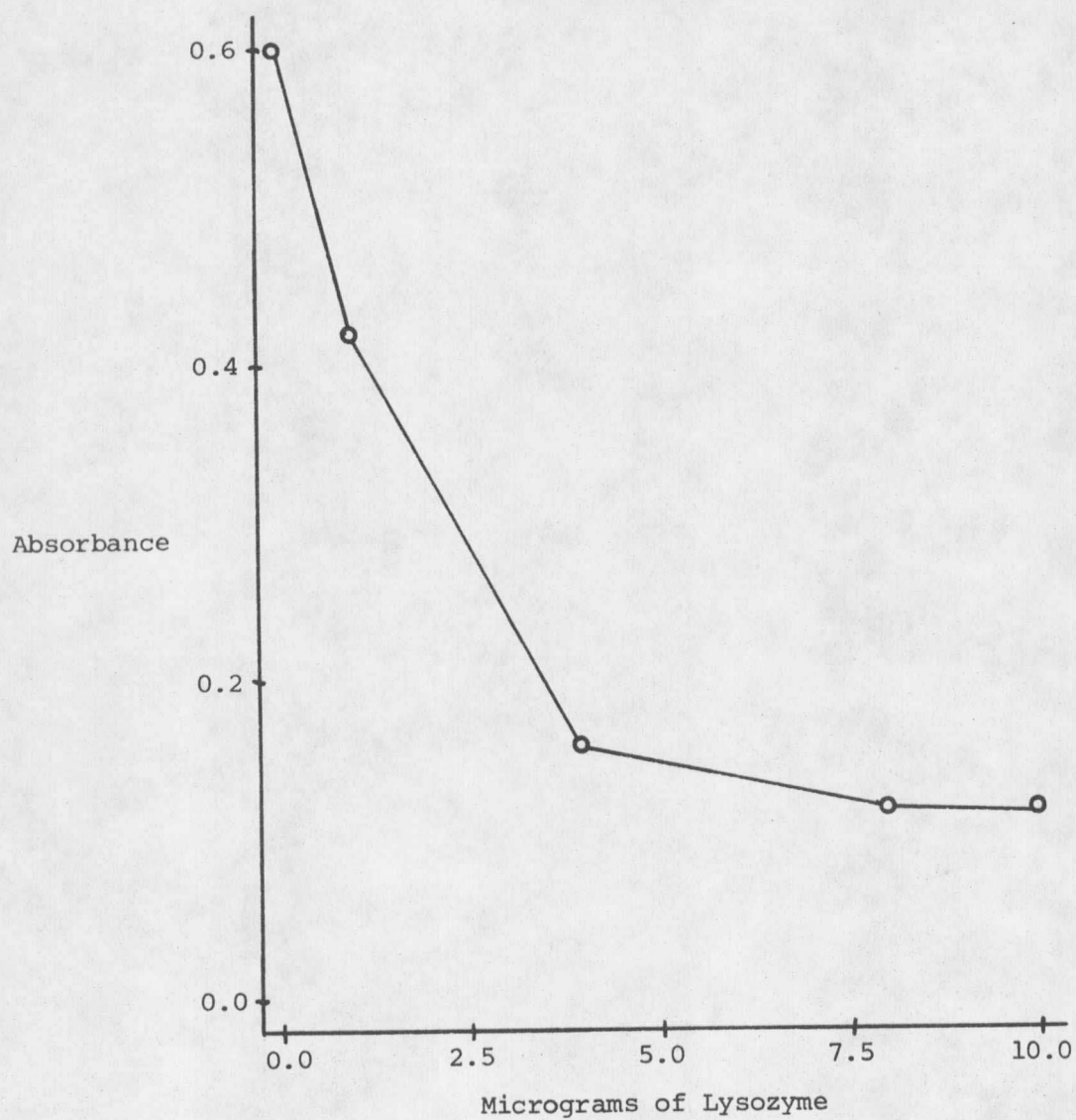


Figure 5. Standardization curve for the lysozyme assay of Smolelis and Hartsell.

values from approximately 1 $\mu\text{g/ml}$ of lysozyme to 10 $\mu\text{g/ml}$. 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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$. Unknowns were usually set to 100 $\mu\text{g/ml}$. 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.

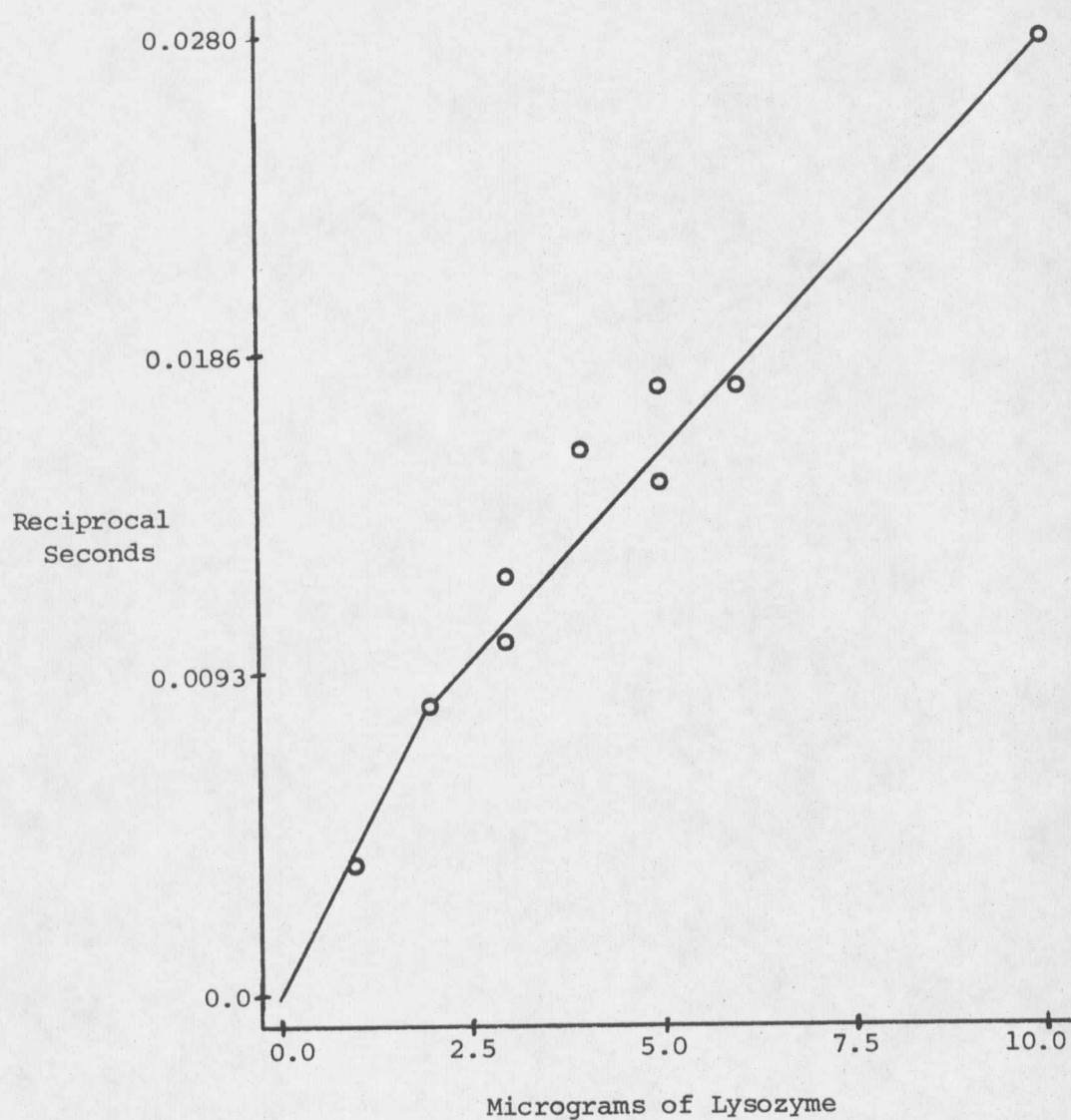


Figure 6. Standardization curve for the lysozyme assay of Gorin, Wang and Papapavlou.

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 X1, 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 $\mu\text{g/ml}$. 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, 0.1g 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:

<u>Material</u>	<u>Supplier</u>	<u>Supplier Code</u>	<u>Lot Number</u>
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 Bio- chemicals 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 coarse 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 μ m 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 μ l syringe equipped with an incremental dispenser (also Hamilton) which permitted increments of approximately 0.2 μ l. 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 μ g/ml to 10 μ g/ml were used. Usually an amount equivalent to 10 μ g of analyte was spotted with drying by a heat gun between 0.2 μ l 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 μ m). 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 μ M in dye and 114 μ M 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 μ g/ml of lysozyme prepared from the stock preparation in (1). The solutions were each diluted to 100 μ g/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 μ g/ml were used. All points except 100 μ g/ml were in duplicate. Four determinations were made at 100 μ g. 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 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). Deuteriophosphoric 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 μ m 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, lyophilized diluted to 100 $\mu\text{g/ml}$ (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 $\mu\text{g/ml}$. 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. Photo-oxidation 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

absorbance from hematoporphyrin at the 650 nm analytical wavelength, no further work was required for assay.

Table 1. Lysozyme Photooxidation Mixtures

Solution	1. Control	2. Azide	3. Deuterium
Lysozyme	30.0 mg	30.8	30.6
Na Acetate	82.0 mg	82.2	83.1
Acetic Acid	62.1 mg	60.6	61.0
D ₂ O	--	--	22.0 g
H ₂ O	20.0 g	18.0	--
0.2 M NaN ₃	--	2.0 ml	--
pH	4.7	4.7	4.7
Solution	4. Control	5. Azide	6. Deuterium
Lysozyme	30.5 mg	30.3	30.2
NaH ₂ PO ₄ ·H ₂ O	207.4 mg	209.5	208.2
Na ₂ HPO ₄	62.4 mg	63.6	63.9
D ₂ O	--	--	22.0 g
H ₂ O	20.0 g	18.0	--
0.2 M NaN ₃	--	2.0 ml	--
pH	6.1	6.2	6.3

All but the phosphate control solution were evaporated to dryness in a rotary film evaporator, taken up in two ml of slightly acidified (HCl) water and the protein was precipitated by addition of 12 ml of acidified (1% HCl) cold acetone. The protein was redissolved in two ml of water and reprecipitated twice, then dried by a gentle air stream. The residue was taken up in two mls of water and half was

hydrolyzed for amino acid analysis by the methanesulfonic acid method and the other half by basic hydrolysis. Amino acid analyzer run codes: phosphate-D₂O acid 813-74, base 814-74; acetate-D₂O acid 815-74, base 820-74; acetate-control acid 816-74, base 819-74; acetate-azide acid 817-74, base 821-74; phosphate-azide acid 818-74, base not run.

Dry State Oxidation of Amino Acids and Proteins by Microwave Generated Singlet Oxygen

The microwave generator and reactor system was used with the following modifications and operating parameters. The Owen-modified Type-C antenna was used. Most experiments were run using pure oxygen with exceptions as noted using carbon dioxide or 10% oxygen in helium. No dry ice trap was used for dry state reactions although a dust trap was used with powdered absorbants. The reactor vessel was either the 3 × 20 cm tube shown in the figure or an 8 × 15 cm 500 ml flask both of which were fitted with 24/40 standard taper joints. After the usefulness of violanthrone as a monitor was discovered (Section C), a violanthrone test spot on filter paper was attached to the inside of the flasks at the top and the gas pressure and power output were adjusted to give the maximum red glow with minimum power use.

A. Thin layer oxidation of amino acids. In a typical experiment two to 20 µgs of the substrate was spotted by means of a Hamilton

microsyringe with an incremental applicator attachment. Typical applications would be 2.5 μ l of a four mg/ml solution. When the substrate was oxidized directly on a suitable chromatographic material, either paper or thin layer sheet, it was developed on the same sheet by a suitable solvent system and visualized as noted. While the substrates mentioned in preceding work on amino acids were all surveyed, most of the work was done with methionine due to the ease with which it can be analyzed for the extent of reaction. Comparison materials were prepared by photooxidation sensitized by rose bengal. Typical preparations were 100 mg of substrate, and five mg of dye in 25 ml of water. The solution was exposed to light for two hours in 50 ml flasks illuminated from the bottom using a 750 watt projector and mirror at room temperature. This gave essentially complete conversion of Met to the sulfoxide. The rose bengal was removed by chromatography on A-25 DEAE Sephadex.

1. Ten μ g of Met was exposed on a 2.5 $\times$ 10 cm strip of Bakerflex silica gel thin layer sheet (with fluorescent indicator) at 5-10 torr, for 15 minutes at 80% power. The strip was developed with butanol-acetic acid-water(4:1:1) (solvent BAW) and visualized with ninhydrin. A control identical except for the exposure was also run.

2. As in (1) with histidine, methionine, N-glycyltryptophan (NGT), alanine and cystine. Exposure was for 20 minutes. Controls were run.

3. A series of strips as in (1) was prepared with 10 μ g spots of Met and N-benzoylhistidine (NBH) (except as noted) using EK 13191 prescribed silica gel sheet with fluorescent indicator. The samples were run at various settings of power and pressure to investigate the parameters of maximum activity. Development was with BAW and visualization, except as noted, with ninhydrin. The following settings were investigated for 15 minutes, except (7) which ran for one hour.

1.	2-3 torr	(pure O ₂)	90% power
2.	2-3		50%
3.	5-6		50%
4.	5-6		25%
5.	15		90%
6.	1		90%
7.	2-5		90%

Sample (6) also had spots of His and tyrosine. Sample (7) used Met and NGT. The NGT was done in duplicate one of which was visualized with ninhydrin and the other by the Ehrlich's reaction. Two samples were also run as in (1) and (6) (Met and NBH only) with the strips just clear of the mercuric oxide deposit near the discharge.

4. Forty μg of Met (10 μl of four mg/ml preparation in water) was spotted on an area of approximately one cm^2 on a glass microscope slide. The slide was exposed at 4-5 torr and 80-90% power for one hour. Using a 10 μl Hamilton syringe, 10 μl of water was spotted on the slide and smeared around. This apparently dissolved the Met film. About five μl was taken back up into the syringe. Spots of 0.6, 1.2 and 2.0 μl were applied to 10 cm prescribed silica gel thin layer chromatographic sheets. The chromatograms were developed with BAW and visualized with ninhydrin.

5. As in (4) using cellulose acetate electrophoresis strips instead of glass for the oxidation support and eluting by drops of water.

6. As in (1) above using alumina (EK) thin layer sheets. Since no data were available on the relative R_f 's of Met and the sulfoxide on alumina, a rose bengal oxidized Met standard was run for comparison. Various solvents were used: BAW; ethanol-water-ammonium hydroxide(7:2:3); and water. Ninhydrin was used for visualization.

7. As in (1) above using MN-300 microcrystalline cellulose thin layer chromatographic sheet. After oxidation spots of the starting material Met and photooxidized Met were added for comparison.

Development was with BAW and 2-propanol-butanone-1N HCl (60:15:25).

Visualization was with ninhydrin.

8. Met was run in two dimensions on Bakerflex 1B silica gel and MN-300 microcrystalline cellulose. Ten cm square sheets were spotted on a corner and exposed for 30 minutes at 1-3 torr and 20% power. The sheets were then developed with BAW, dried and reexposed under the same conditions. The sheets were then developed with BAW at right angles to the first run and visualized with ninhydrin.

9. As in (7) using thin layer plates prepared by covering microscope slides with a slurry of Carboxymethyl cellulose (Biorad) in dilute acetic acid and drying. Only BAW was used for development.

10. As in (1) using Whatman Paper Chromatography paper with Met and photooxidized Met standards spotted after exposure. Developed with BAW in descending mode in a large chromatographic jar.

11. As in (1) and (7) (BAW development only) using CO₂ gas in the discharge. Conditions were adapted from Gollnick (Gollnick and Schade, 1973). Two torr at 50% power for 25 minutes.

12. As in (1) using 10% O₂ in He for 15 minutes at 15 torr and 50% power.

13. Met was photooxidized by sensitizing with rose bengal in the dry state. Paired 10 μ g spots of Met were placed on two apiece of EK 13191 prescribed silica gel and MN-300 cellulose sheets. One μ g of rose bengal was spotted on top of one Met spot on each sheet. One sheet apiece of each type was illuminated for 40 minutes by a projector using a DDB 750 watt bulb operated at 80% power. The other sheet was kept in the dark. Development of all sheets was with BAW. Visualization was with ninhydrin.

B. Oxidation of proteins in the dry state: lysozyme

1. An attempt was made to oxidize lysozyme. Thirty mg of lysozyme (Sigma Grade I cat. no. L6876 lot 93C-8000) was dissolved in 25 ml of doubly distilled water. A 1:10 dilution of this had an absorbance at 280 nm of 0.300, equivalent to 1.15 mg/ml in the original preparation. Aliquots of 2.5 ml of the original preparation were added to each of four 500 ml lypholyzer flasks. The solutions were lypholyzed to nice fluffy masses of enzyme. The masses were exposed to the singlet oxygen stream for 1, 5, 10 and 22 minutes. Settings were 3-5 torr at 50% power. The exposed enzyme was taken up in 25 ml of buffer LZ and assayed for activity by the Gorin method. M. lysodeikticus cells preparation was 45 mg in 200 ml of LZ buffer. Standards were prepared by diluting the 1:10 dilution prepared above 1:10, 2:10, 2:5, 3:5 and 4:5 in LZ buffer.

2. Essentially the same as in (1) except the oxidations were continued for up to six hours. Sixty mg of lysozyme were dissolved in 50 ml of water. A 1:10 dilution of this had an absorbance of 0.298 at 280 nm equivalent to 1.13 mg/ml in the stock solution. A 10 ml aliquot of the stock solution was placed in each of four, 500 ml lyophilizer flasks, frozen and lyophilized. These were lyophilized at least four hours past apparent dryness. The fluffy lysozyme masses (11.3 mg apiece) were exposed at three torr and 20% power (violan-throne tuning) for 0, 1, 2 and 6 hours. The lysozyme in each flask was taken up in about 25 ml of water and 2.5:10 dilutions in LZ buffer were prepared (3.0:10 for the six hour sample). The absorbances at 280 nm were determined. These dilutions were used in the Gorin activity assay. Standards were prepared by diluting the original 1:10 dilution of the stock solution 2:10 and 5:10 in LZ buffer. Substrate was prepared by suspending 25 mg of *M. lysodeikticus* cells in 100 ml of LZ buffer.

3. Essentially as in (1) using a cellulose absorbant support. Thirty mg of lysozyme were dissolved in 25 ml of water. The absorbance at 280 nm of a 1:10 dilution was 0.317; equivalent to 1.2 mg/ml in the stock solution. One g apiece of cellulose chromatographic powder (Excorna) was placed in two 500 ml lyophilizer flasks and 2.5 ml of the lysozyme preparation was added. Twenty ml of water

was added and the pasty masses were frozen and lyophilized. One of the powder-enzyme preparations was exposed for 70 minutes at five torr and 20% power. The enzyme was washed off the cellulose in 50 ml of LZ buffer. The solutions were filtered through 0.45 μ m HAWP Millipore filters. The absorbances at 280 nm were determined. The dilution of the original preparation, used for absorbance measurement, was used as a standard and compared to the assay results from (1).

4. Essentially the same as (3) using microcrystalline cellulose (Sigmacell) as an absorbant. Lysozyme (45 mg) was dissolved in 25 ml of water. Absorbance at 280 nm of a 1:10 dilution of the stock preparation was 0.468, equivalent to 1.75 mg/ml in the stock. Ten g of Sigmacell microcrystalline cellulose was washed with water, 10% acetic acid then water again. The cellulose was left moist and 12.5 ml of the lysozyme preparation was added. The moist mass was frozen and lyophilized. The preparation weighed 7.5 g at this point. One third (2.5 g) was eluted with LZ buffer to give 24 ml of a solution with $A_{280} = 0.367$ coded control 1. The other 2/3 was exposed for 40 min at 12 torr and 20% power using 10% oxygen in helium. After exposure this was eluted using LZ buffer to give 38.5 ml of solution, $A_{280} = 0.630$; coded Run 1. The experiment was repeated using 12.5 ml of the stock preparation and five g of unwashed Sigmacell. Note: When Sigmacell without lysozyme was washed with LZ buffer, the

absorbance of the solutions were less than 0.05 after filtering. A few ml of water was added to make a thick slurry and the mass was lyophilized while the lyophilizer flask was kept immersed in a container of water. This was to be sure the lysozyme would be dried to the cellulose and not be in free crystals. Three g of the dried powder were exposed for one hour and eluted as above. The other two g were eluted as above. The solutions gave, for Run 2, 23.0 ml of $A_{280} = 1.20$ and Control 2, 21.3 ml of $A_{280} = 0.748$. All four solutions were diluted to approximately 100 $\mu\text{g/ml}$ with LZ buffer and the absorbance was rechecked. The samples were then assayed for activity by the Gorin method. The 1:10 dilution of the stock preparation was also assayed. Cells were prepared by suspending 58 mg in 250 ml of LZ buffer. Standards were prepared by dissolving 36 mg of lysozyme in 25 ml of water. A 1:10 dilution in LZ buffer had $A_{280} = 0.440$. The dilution contained 165 $\mu\text{g/ml}$ of lysozyme. Dilutions of this of 1, 2, 3, 6 and 8 to 10 were prepared.

5. Essentially the same as (3) using silica gel as an adsorbant. Aliquots of 2.5 ml of the stock solution prepared in (3) (1.2 mg/ml) were added to two 500 ml lyophilizer flasks containing one g apiece of silica gel powder chromatographic adsorbant (Baker, 60-200 mesh, lot 43687). The pasty masses were frozen and lyophilized. One sample was exposed for 75 minutes at five torr and 50% power with

stirring. The other was used as a control. The silica gel preparations were eluted with 25 ml apiece of LZ buffer. The solutions were filtered through HAWP 0.45 μ m Millipore filters. The absorbances at 280 nm were 0.054 and 0.058 for the control and oxidized samples, indicating essentially no removal of protein. Enzymatic activity, as determined by Gorin method, was essentially absent. The preparations were then eluted with 25 ml of 80% acetic acid. The solutions were not filtered. Absorbances at 280 nm were 0.323 and 0.397, respectively, for the control and oxidized material. Some silica gel was present as the solution had a hazy appearance. Absorbances at 570 nm were 0.042 and 0.078, respectively. Absorbances of silica gel suspensions have a fairly uniform absorbance from 200 nm through the visible. The lysozyme solutions in 80% acetic acid were assayed by the Gorin method, as described, by adding 0.1 ml directly to three ml of the substrate suspension. No activity was detectable.

6. As in (5), with one hour of exposure, except that the silica gel was eluted with 25 ml of 33% acetic acid. The solutions were filtered through HAWP 0.45 μ m Millipore and lyophilized. The lyophilized powders were taken up in LZ buffer and allowed to set several hours to renature. Absorbances at 280 nm were 0.312 and 0.118 for the control and oxidized, respectively. The solutions were assayed for enzymatic activity by the Gorin method. Substrate was

prepared by suspending 22 mg of cells in 100 ml of LZ buffer. Standards were prepared by diluting the stock solution 1:10 and 1:25.

7. As in (6) using 2.5 ml apiece of a lysozyme preparation of 60 mg in 50 ml of water on one g of silica gel. The silica gel used was prewashed with 80% acetic acid, and water then oven dried until just moist. The oxidized sample was exposed for 30 minutes at two torr and 40% power. After lyophilizing to remove the 33% acetic acid, the precipitates were taken up in 25 ml of water and assayed for activity. A new preparation of cells, 22 g in 100 ml of LZ buffer was used. Activity standards were prepared by diluting the above stock solution 1:10 and 1:25 with LZ buffer. The remainder of the 25 ml water solutions of lysozyme were lyophilized and hydrolyzed for 22 hours in two ml of 2 N methanesulfonic acid as described. The hydrolysates were neutralized with 4 N sodium hydroxide, then 0.5 ml of saturated citric acid was added, giving a pH of about 2.0 (pHydrion paper). The oxidized sample was diluted to five ml with 2.2 citrate loading buffer. Note: This leaves the solution high in Na^+ ion. Amino acid analyzer run codes were: for control 711-74 and the oxidized 709-74. A control of fresh lysozyme was prepared by hydrolyzing, as above, four mg and diluting in the last step to 10 ml with loading buffer. Run code 708-74.

8. Essentially the same as (6) except that the total amount of enzyme was considerable larger than previous runs. This was to provide enough product to analyze both acid and base hydrolyzates for amino acid content.

Silica gel was prepared by washing it with benzene, toluene, chloroform, acetone glacial acetic acid and water than heating in a sterilizer drying oven (temperature greater than 160°C) for 15 hours. The material was then allowed to stand, covered but available to the air, for a day to reduce the surface activity by partial rehydration. Three g portions of this were used here, and in section (10) following with ribonuclease, in each batch. Thirteen ml apiece of a 1.46 mg/ml solution of lysozyme (19 mg total) were added to two of the three g batches of silica gel. The preparations were frozen and lyophilized. One preparation was exposed for 0.5 hour at three torr and 40% power in the reactor. The other was maintained as a control. The lysozyme was eluted from the silica gel with 33% acetic acid. The solvent was lyophilized away. The residue was taken up in a few ml of water and filtered through a HAWP 0.45 μ m Millipore filter. The solutions were made up to 10 ml with water and the absorbance of a 1:10 dilution in LZ buffer was determined. This was 0.350 for the oxidized and 0.412 for the control, equivalent to recoveries of 13.2 and 15.5 mg of lysozyme. The enzymatic activity by the Gorin method and protein concentrations by the Lowry method were determined on this diluted solution.

The Lowry values for the yields were 12.0 and 14.9 mgs, 63 and 78%, respectively.

The violanthrone tuning spot inside the reactor was extremely dim during this run. The presence of three g of silica gel seems to be very quenching. To check the singlet oxygen output, after the silica gel was removed the reactor was started again at the same settings and gave a good bright red reaction.

The remaining oxidized and control solutions were dried on a rotary film evaporator and taken up in two ml of water. One ml apiece of each solution was hydrolyzed for amino acid analysis by basic and acidic hydrolyses. The run codes were: 806-74, 807-74, 808-74 and 810-74 for acidic and basic hydrolyses for the oxidized and control samples, respectively.

9. Ribonuclease-A. Essentially the same as (5) using silica gel as an adsorbant. Silica gel was washed with water and acetic acid, then water again, decanting the fines each time. The material was oven dried at 230°. About 25% of the material was lost as fines. Ribonuclease-A, (Sigma No. R-5125, lot 99B-8020 bovine pancreas), 25.3 mg, was dissolved in 11 ml of water. One ml of this when diluted to five ml had an absorbance at 278 nm of 0.284. The dilution was further diluted 1:25, 1:50 and 1:100 with 0.1 M pH 5 acetate buffer to furnish standards for enzymatic activity analysis.

The remaining 10 ml of the stock solution was added to the silica gel with enough water (about 15 ml) to make a thick slurry. The slurry was frozen and lyophilized yielding eight g of dried mixture. Four g of this were exposed for one hour at 40% power and three torr, with stirring. The enzyme was desorbed with 80% acetic acid from the exposed sample and the other four g of silica gel which was used as a control. The silica gel was first eluted with water until the fine silica produced by stirring was removed then eluted with the 80% acetic acid. The eluent was collected in six 8.0 ml fractions, whose absorbances at 278 nm were checked. The fractions which had the greatest absorbances were combined up to a volume of 25 ml in each case. Some non-protein material was present as recoveries based on A_{278} were 120-150%. One ml of each was diluted to 10 with water containing 0.3 ml of 19 M sodium hydroxide to give a buffer of approximately equimolar acetate-acetic acid and a pH of approximately 5.0. These, and other similar solutions were allowed to set overnight after diluting to renature. These dilutions were used for enzyme activity assays. The rest of the 80% acetic acid solutions were dried on a rotary film evaporator, taken up in 25 ml of water and analyzed for protein content by the Lowry method. The analyses took two mls apiece of the solutions, the rest of which were redried on the rotary film evaporator. The residues were taken up in 2-4 ml of 6 N (constant boiling) hydrochloric acid, sealed in glass ampules and heated for

24 hours at 110°C. Note: There is no Trp in ribonuclease so hydrochloric acid offers some advantages here over methanesulfonic acid. It can be removed by evaporation so less problem with salt is encountered. The samples were opened after heating, evaporated in a vacuum over desiccant and sodium hydroxide pellets and taken up in 10 ml of pH 2.2 amino acid analyzer loading buffer. Analyzer sample codes were oxidized material: 783-74, control 784-74. A control was prepared to test the effect that solution in 80% acetic acid has on the enzymatic activity of ribonuclease. Two mg of the enzyme was dissolved in five ml and allowed to stand eight hours at room temperature. The absorbance at 278 nm was 0.266. A 1:25 dilution of this was prepared for activity analysis. One ml of the 80% acetic acid solution was diluted with distilled water containing 0.3 ml of 19 M sodium hydroxide to give a pH 5 buffer. The Kunitz ribonuclease assay was used.

10. Essentially the same as (5) and (9). This determination with ribonuclease-A was run parallel with the lysozyme work described in (8) and used the same silica gel preparations and reactor exposures. Ten ml apiece of 2.0 mg/ml (52.5 mg/20 ml) solutions of ribonuclease-A were added to the three g batches of silica gel which were lyophilized and exposed as in (8). The enzyme was a new batch from Sigma, number R5125 (same as in (9) above) but lot number

122C-8220. The control enzyme was eluted with 80% acetic acid and the oxidized was eluted by stepwise portions of 33, 50, 66 and 80% acetic acid. The best results seemed to be obtained with the latter method, as most of the absorbance at 280 nm was eluted at 33% and in the initial part of the 50% where the concentration was rising. Little absorbance was eluted in the later part of the 50% or with stronger solutions. The solvent was lyophilized off and the residues were taken up in a few ml of water and filtered through HAWP 0.45 μ m Millipore filter. The solutions were made to 10 ml and the absorbances at 280 nm was determined. They were 0.538 and 0.572 for the oxidized and control, respectively.

This is equivalent to 8.7 and 9.2 mg total recovery. Lowry determinations were made of protein concentrations. These gave values of recovery of 6.7 and 7.3 mg, 34 and 37%, respectively.

The Kunitz activity assay. A different lot of yeast ribonucleic acid (Sigma number 6750, lot 14C-8010) was used for the substrate (26 mg/40 ml). Standards were prepared by diluting the original 2.0 mg/ml preparation 1:50, 1:200 and 1:500 to give 42, 10.5 and 4.2 μ g/ml solutions. The oxidized solution was diluted 1:10 to give 67 μ g/ml and the control was diluted 1:40 to give 18 μ g/ml solutions. Both gave enzymatic activity assays equivalent to 21 μ g of activity in the original solution. These were, respectively, 31 and 117% active.

The remaining stock solutions were dried on a rotary film evaporator and taken up in two ml of water. One ml apiece of each solution was hydrolyzed for amino acid analysis by basic and hydrochloric acid hydrolyses. The run codes were: 802-74, 803-74, 804-74 and 805-74 for acidic and basic hydrolyses for the oxidized and control samples, respectively.

As there was some question about the amino acid analysis of the above treated samples, a hydrolyzate was prepared of 2.1 mg of enzyme fresh from the bottle in lot 123C-8220. Hydrolysis was performed for 23 hours in one ml of fresh 12 N hydrochloric acid plus one ml of doubly distilled water. After drying, the residue was taken up in 10 ml of loading buffer, run code 822-74.

C. Violanthrone. Ogryzlo (Ogryzlo and Pearson, 1968) described several reactions involving the brilliant chemilumescence induced by the quenching of singlet oxygen by violanthrone.

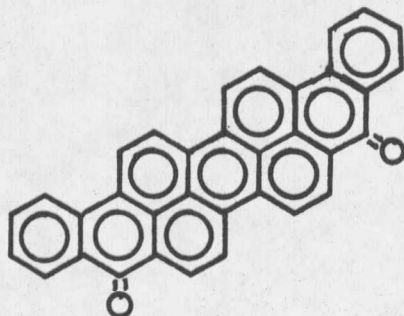


Figure 7. Violanthrone

Several solvents were tried. The dye is essentially insoluble in water. There is very slight solubility in ethanol and acetone. It has somewhat better solubility in benzene. It seems to be readily soluble in dimethylphthalate, but as this is not volatile, it was not very useful. Chloroform proved to be a convenient solvent. About 10 mg of the dye was dissolved in 25-30 ml of chloroform. This yielded a purple solution with a reddish fluorescence.

1. A few tenths of a ml of this were dried on glass and exposed to the microwave generated oxygen stream.

2. As in (1) but using absorbants to disperse the dye.

The following materials were surveyed:

- Silica gel TLC Sheets (EK 6060 and Bakerflex 1B)
- Paper Chromatography Paper (also other filter papers)
- DEAE-Cellulose powder in bulk (Biorad Cellex-D)
- CM-Cellulose powder in bulk (Biorad Cellex-CM)
- Silica Gel-H powder in bulk (Merck)
- Alumina TLC sheet (EK)
- Microcrystalline Cellulose TLC Sheets (MN-300)

When a sheet was used, the material was applied as an ink to give good dispersion.

3. Using a small piece of filter paper, with an "X" of violanthrone, placed in the reactor flask, various power settings were tried for pressure and power to optimize singlet oxygen production in the system. A tuning spot like this was used in all of the later work.

Use of the Clark-Type Electrode to Demonstrate Photooxidation

A number of experiments were run using a Clark-type (Clark, 1956) membrane oxygen electrode to determine the presence of oxygen consuming photooxidation. The theory of operation is that oxygen is allowed to diffuse through a plastic membrane, usually Teflon or polyethylene, the former being more common, into an electrode chamber. The oxygen is then reduced to peroxide by impressing a voltage of 0.6-0.8 v across the electrodes, which are usually noble metal. The current observed in the electrode circuit is proportional to the amount of oxygen present. The solution in the electrode chamber is a buffered (often bicarbonate) potassium chloride electrolyte. The volume actually in contact with the cathode is kept as small as possible to minimize electrode sluggishness. This is usually accomplished by stretching the membrane over the cathode, allowing only a film of the electrolyte to be under it. The process of oxygen diffusion through the membrane is influenced by both temperature and the degree of saturation with oxygen of the solution being analyzed. The diffusion has a temperature coefficient of about 4%/°C. This represents a kinetic effect. The amount of oxygen diffusing is also affected by an equilibrium effect with degree of saturation. This directly influences the partial pressure of oxygen in equilibrium with the solution. The higher the equilibrium partial pressure, the faster the oxygen will diffuse through the membrane. This is actually what

is measured, not the absolute concentration of oxygen. Since salinity and temperature strongly influence the solubility of gases in liquid, these factors have to be allowed for in calibration.

The instrument used in these studies was manufactured by Delta Scientific, Lindenhurst, N.J. It is their Model 110-1 membrane probe used with a Model 106 Dissolved Oxygen Analyzer. The power source in this is a pair of size D flashlight batteries. The electrode probe consists of the membrane (Teflon) over a gold button cathode with a silver anode. This particular instrument was never intended for precision research work, being constructed for use in the field as a ground water dissolved oxygen meter. In addition, it seems to have a rather high background current which is somewhat capricious. Perhaps this is why the unit was sold as surplus by its previous owners. However, the unit proved to be very able to demonstrate the presence or absence of an oxygen consuming reaction and gave an idea of its relative rate, although it would be difficult to use for any precise kinetic studies. Most of the work detailed here was intended to be preliminary experiments with more detailed work to be done later on a research instrument.

The instrument was calibrated against air saturated water at various temperatures to determine scale response and the zero-oxygen current--background current--was determined on solutions of 5% sodium sulfite. The background current varied from 3-15 relative scale

values (rsv). Saturated values at room temperature (23-30°) were 150-180 rsv and seemed fairly reproducible at any given temperature.

A. Studies of amino acids. The samples were in a 125 ml erlenmeyer flask with a 24/40 ground glass joint. This joint does not exactly fit the taper of the probe, which was designed for the taper of a standard 500 ml biological oxygen demand Winkler flask, but, with careful fitting, will give an adequate seal. Care must be taken that no bubble of air or opening which would permit inward diffusion of oxygen remains. The flask was illuminated from the side (rapid stirring by a magnetic stir bar is essential) by one of two different lights. Experiments 1-5 used a 150 watt G.E. spot lamp. Later experiments used a 750 watt project operated at 80% power. The temperatures were maintained at near room temperature by a large beaker water bath with no additional cooling. The temperatures generally rose from 2-3°C during the experiment.

1. Rose bengal with histidine

a. Water was vigorously stirred open to the air for one half hour, then the probe was inserted and sealed. A reading of 170-176 rsv (85-88 × 2 range) was observed to hold steady for an hour.

b. One g of dipotassium hydrogen phosphate monohydrate was added (solution ca. 0.03 M) to serve as a buffer ca. pH 9.0-9.2.

Oxygen readings were 172-176 ($86-88 \times 2$) for 30 minutes while illuminated.

c. His (50 mg, 3×10^{-4} mole, 2.5×10^{-3} M) was added.

Readings were 172-176 ($86-87 \times 2$) for 40 minutes while illuminated.

d. Two mg of rose bengal was added in the dark. Readings were 172-176 ($86-88 \times 2$) for 20 minutes.

e. The light was turned on giving the consumption shown in the "Results" section. Oxygen consumption was clearly evident in one minute. The light was turned off for noted periods of time with the noted leveling off.

2. As in (1) with cysteine instead of His. The dye, substrate and phosphate were all mixed in the dark. Temperature was about 22°C . The readings were essentially constant for 15 minutes at 150 (75×2) rsv. Light dependent oxidation was observed within 10 seconds.

3. As in (1) with His and two mg of crystal violet. Temperature was about 30° . In the dark for 15 minutes, the readings rose from 176 to 184 ($88-92 \times 2$).

4. As in (3) with CysH instead of His. Dark readings were stable at 162 (81×2) for 30 minutes.

5. As in (4) with 15 mg of CysH and 50 mg of crystal violet. Readings went from 156 to 161 ($78-80.5 \times 2$) in 63 minutes in the dark.

6. As in (4) with the brighter light.

7. As in (3) with His except that two mg of proflavin was used instead of crystal violet. Stable in the dark for 20 minutes.

8. As in (2) with three mg rose bengal and 50 mg of cystine (Cys₂) instead of cysteine (CysH). The readings rose from 152 to 168 in 45 minutes in the dark and rose to 186 in another 50 minutes in the dark before the light was turned on.

9. As in (8). The dye and phosphate solution were stirred while illuminated for 40 minutes while the oxygen readings rose from 136 to 166. At the same time, the water-bath temperature rose from 20 to 32°. Fifty mg of cystine was added and kept in the dark for an hour. The readings rose 168 to 188. The temperature was at 31°C.

10. The same solution used in (9) was reoxygenated by stirring vigorously for three hours while open to the air in the dark. It was then re-illuminated. At the end of the second illumination, the rose bengal had faded to a pale yellow.

11. The same solution as in (9) and (10) was reoxygenated again, but the dye remained faded. Two mg of rose bengal was then added and the oxygen reading in the dark was stable at 179 for 30 minutes.

12. As in (1) with rose bengal and alanine instead of His. The readings were stable at 192 for 15 minutes in the dark at a temperature of 33°.

13. This experiment used a smaller, 50 ml, flask than the previous one. The substrate was 10 mg of methionine with 10 mg of rose bengal in water. The reaction was run using a 500 watt projector and the solution was in an ice bath. The reading varied $\pm$ one unit from 99 in 10 minutes before the light was turned on.

B. Photooxidation of pyrocatechol

1. A solution of five mg of pyrocatechol (1,2 benzenediol) in water with 10 mg of rose bengal was run as in (A-13) above. An ice bath was used and the solution registered 76-77 for six minutes before starting the light.

2. In a repeat of (1), 11 mg of pyrocatechol was stirred in an ice bath in the dark and held at 98 for seven minutes. When the light was turned for eight minutes, the reading rose from 98 to 102. Ten mg rose bengal was added in the dark with a change from 102

to 104 in 14 minutes. A scale adjustment was made to 100 even and the light was turned on.

Spectral Studies of the Interaction of Hematoporphyrin and Lysozyme

Jori et al. (1971) have reported a peculiar and rather specific dye sensitized photooxidation of lysozyme with hematoporphyrin. They also reported that hematoporphyrin formed a complex with lysozyme which shifts the absorption spectrum of the dye. This shift was from a peak of 390 nm to 408 nm with a slight decrease in peak absorptivity. To attempt to verify this spectral effect, the following determinations were made:

A. Sixty mg of hematoporphyrin dihydrochloride (Cal biochem No. 3731 lot 901390) (8.9×10^{-5} mole) was dissolved in five ml of ethanol (1.78×10^{-2} M). Twenty-two mg of lysozyme (Sigma 3 $\times$ recrystallized lot no. unavailable) was dissolved in 10 ml of 0.1 M pH 4.8 acetate buffer. The absorbance at 280 nm for a 1:10 dilution was 0.503, equivalent to 1.9 mg/ml (1.32×10^{-4} M) in the original solution. Ten μ l of the hematoporphyrin (1.78×10^{-7} moles) was added to two ml of the lysozyme stock solution (2.64×10^{-7} moles). The mole ratio was about 1.5 lysozyme to 1.0 hematoporphyrin. The solution proved to be too dense for spectral scanning in 1.0 cm cells and was diluted 1:2 to 66 μ M lysozyme with the acetate buffer and scanned using a Cary 14. An hematoporphyrin spectrum at the same

concentration in the same buffer was also scanned. TLC determinations of the dye uniformity were run. Forty μg spots of the dye on EK 13191 silica gel sheets were developed with methanol and acetone and appeared homogenous.

B. Using the same source of lysozyme and a new source of hematoporphyrin, the spectra were redetermined at pH 6.2 in buffer LZ (0.1 M sodium phosphate, 10^{-3} M EDTA). Ten μM lysozyme was prepared by dissolving 20 mg in 10 ml of LZ buffer. A 1:10 dilution of this material had an absorbance at 280 nm of 0.487. The stock dilution was diluted 10:12.93. This was rechecked at 280 nm (1:10 dilution), $A = 0.386$, equivalent to 103 μM . Hematoporphyrin was prepared by dissolving 20 mg (3×10^{-5} moles) of the dihydrochloride (Sigma, No. 111875, lot 032C-1370) in 10 ml of ethanol. This was diluted 1:10 with ethanol to give a 300 μM solution. Spectra were obtained on solutions prepared by mixing 0.1 ml of the dye (3×10^{-8} mole) with 3.0 ml of the lysozyme (3.1×10^{-7} mole) or 3.0 ml of a 1:10 dilution of the lysozyme (3.1×10^{-8} mole) or 3.0 ml of the buffer. These gave 1:10 and 1:1 dye:lysozyme ratios.

C. As in (B) except that the lysozyme was prepared in doubly distilled water to a concentration of 10.0 μM to see if the presence of the buffer influenced binding. The pH of the dye lysozyme mixture was 5.4.

D. The experiment was repeated with new sources of both lysozyme and dye. Lysozyme (Sigma Grade I No. L6876, lot 93C-8000) was prepared by dissolving 13.4 mg of dihydrochloride (Fluka purum grade 51250-701459) (2.0×10^{-5} mole) in one liter of 10^{-4} M sodium hydroxide. The pH of this solution was 7.9. It was clear although a very small insoluble residue remained. This is referred to as the stock solution.

1. The absorption spectrum of a 1:2 dilution (10 μ M) of the dye stock was determined. The pH of the dilution was 7.2. The pH was adjusted to 5.2 with solid sodium dihydrogen phosphate and the spectrum was rerun.

2. A solution that was 10 M in dye and 20 M in lysozyme, pH 8.5 was prepared by mixing 12.5 ml of the dye stock with 4.37 ml of the lysozyme stock and diluting to 25 ml. A spectra were determined for this and a pH 5.2 solution, adjusted by adding sodium dihydrogen phosphate.

3. The spectrum was determined for a dilution of 12.5 ml of the dye stock with 12.4 ml of 0.1 M sodium hydroxide.

4. As in (3) with 12.4 ml of acetic acid instead of sodium hydroxide. It was necessary to dilute this solution 1:4 with 50%

acetic acid to determine the spectrum due to peak sharpening which gave a narrower peak of higher absorbtivity.

5. As in (3) except that the dye stock was diluted 12.5 ml to 25 with the lysozyme stock (10 μ M:57 μ M) pH 6.5.

6. As in (3) except the dye stock was diluted 12.5 ml to 25 with water containing 0.15 g of sodium dihydrogen phosphate (0.05 M final concentration), pH 4.8.

Oxidation of Tryptophan by Singlet Oxygen Generated by Non-Dye Means

Tryptophan was chosen for this work because it can be easily analyzed for the extent of reaction by comparing the ratio of light absorbances at 260 and 280 nms. Additional information can be obtained by comparing the absorbance at 360 nm to that at 280 nm. Spectra of Trp and its sensitized photooxidation products were determined for comparison. Trp has an absorbance peak at 280 nm with the ratio of this peak to its absorbance at 260 nm being about 1.47. N-Glycyltryptophan (NGT) also has a maximum at 280 nm with a 280/260 absorbance ratio of 1.65. This latter ratio is also typical of lysozyme. The product of rose bengal sensitized photooxidation of NGT has absorbance peaks at 260 and 320 nms and a minimum at 280 nm with the 280/260 ratio of 0.224. This presumably N-glycyl-N'-formylkynurenine. Kynurenine (Kyn) (obtained from Sigma as the

DL-sulfate, catalog number K-3625, lot number 79B-1120) has absorbance peaks at 256 and 360 nm and a minimum at 280 nm. Kyn has a 280/260 absorbance peak ratio of 0.07.

A. Rose bengal sensitized photooxidation of N-Glycyltryptophan

N-Glycyltryptophan, 1.3 mg (5 μ mole), was dissolved in 50 ml of distilled water. The ultraviolet absorption spectrum of this was obtained using the Cary-14 and absorbances at 240, 260, 280 and 360 were recorded using the DU-2. Five mg of rose bengal was added and the solution was illuminated for 3.5 hours. The solution was stirred by a slow stream of filtered air. Illumination was provided by a Kodak Kodalight Projector, Master Model Series 1 using a DDB 750 watt bulb. Line power was reduced to 80%, approximately 95 volts, by means of a Staco autotransformer to lengthen bulb life.

At the end of 3.5 hours, the solution was filtered through a bed of one g of A-25 Sephadex (course), in a three cm diameter column fitted with a glass frit disc, to remove the dye. The Sephadex bed was about two cm deep. The spectrum and particular absorbances were determined as with the starting material. The solution was lyophilized and the dry material was then taken up in one ml of distilled water. A sample, ca. 0.2 ml, was saved for thin layer chromatography and the rest was placed in a Carius tube with an equal volume of fresh 12 N hydrochloric acid. The tube was degassed by three freeze-thaw cycles

while under vacuum, sealed and placed in an oil bath at 108° for 24 hours. After hydrolysis, the tube was opened and the contents placed in a 15 ml flat bottomed bottle. The solution had a distinct yellow color. The bottle was placed in a vacuum desiccator with desiccant and sodium hydroxide under vacuum for overnight. The residue was taken up in about 0.8 ml of distilled water and about half the sample was saved for thin layer chromatography and amino acid analysis. The remaining half was diluted to 10 ml in distilled water and its spectrum and specific absorbances at the selected wavelengths were determined. About a third of the sample saved for amino acid analysis (about 0.1 ml) was diluted to 1.5 ml with pH 2.2 citrate loading buffer and analyzed by the Beckman automatic amino acid analyzer (Run code 734-74). The sample was run only on the shortest column using pH 5.24 buffer. An authentic sample of Kyn was also run for comparison on the Cary-14, DU-2 and amino acid analyzer. The Kyn solution had a distinct yellow color. Thin layer chromatographic comparison was made of NGT, its sensitized photooxidation product, the hydrolyzate of the product, Trp, Kyn and Gly as described in the section on thin layer chromatography of amino acids. Fluorescence, fluorescent quenching, ninhydrin and Ehrlich's reagent were used for visualization.

B. Oxidation of tryptophan by hydrogen peroxide-hypochlorite generated singlet oxygen. Ten ml of a solution 0.025 g of Trp

(1.2×10^{-4} mole) in 0.45 ammonium chloride pH 9.5 buffer was mixed with 0.13 ml of 30% hydrogen peroxide (1.2×10^{-3} , 10 molar excess over Trp) at ice bath temperature. Sodium hypochlorite (Purex: 5% or 0.68 M sodium hypochlorite), 1.1 ml (4×10^{-4} mole, 3 molar excess over Trp) was added dropwise while cold. The solution was allowed to stand cold for one half hour, then diluted 1:200 with 0.045 ammonium chloride buffer. Absorbances at 260 and 280 nms were determined. For comparison, the absorbances of a control solution of Trp with only the peroxide was determined.

C. Oxidation of tryptophan and lysozyme by potassium perchromate generated singlet oxygen

1. Preparation of potassium perchromate. Potassium perchromate (peroxychromate), K_3CrO_8 or $K_3CrO_2(O_2)_3$, was prepared as described by Peters et al. (1972). A cold solution of 16.9 g of potassium chromate (0.087 mole) and 3.5 g of potassium hydroxide (0.065 mole) in 200 ml of water was added slowly to a cold solution of 100 ml of methanol, 200 ml of water and 30 ml of 30% hydrogen peroxide (0.26 mole). A red-brown powder precipitated. This was filtered off, washed with water and cold methanol and dried by briefly pulling air through it on the filter. The preparation was stored at $-25^\circ C$ (inner cold room) in a desiccator. Yield was 2.5 g (0.0084 moles, 9.8% of theoretical based on potassium chromate). This material appeared to

be nearly insoluble in water. When it was added to water, a very faint yellow solution resulted which probably was from a small amount of the chromate being present. When allowed to warm and stand in water, oxygen was evolved, the precipitate dissolved and the yellow chromate color formed.

2. Oxidation of tryptophan. Ten ml of 1.2×10^{-2} M Trp (1.2×10^{-4} moles) in distilled water in an ice bath was mixed with 10 ml of 2 M pH 9 ammonium chloride buffer and 25 g (8.4×10^{-5} mole) of potassium peroxychromate. The solution was allowed to warm slowly and sat overnight. The solution was diluted 1:100 in 0.2 M pH 9 ammonium chloride buffer and the 260 and 280 nm absorbances were determined.

A control was prepared in the same manner except that instead of the peroxychromate, 25 mg (1.3×10^{-4} mole) of potassium chromate was added to test the effect of chromate on Trp. A blank was prepared consisting of 25 mg of potassium chromate in 20 ml of one molar pH 9 ammonium chloride buffer. A Trp solution of the same concentration but without the chromates was used as a standard. These last three preparations were allowed to stand overnight next to the peroxychromate-Trp solution, then diluted 1:100 in 0.2 M ammonium chloride buffer and the 280 and 260 nm absorbances were determined.

3. Oxidation of lysozyme. Lysozyme, 0.286 g, (2×10^{-5} mole, Sigma 3 $\times$ recrystallized grade) was dissolved in 10 ml of distilled water and filtered, using a Swinney adaptor, through a 0.45 μ m Millipore filter. One ml of this preparation (2×10^{-6} mole) was cooled on an ice bath and mixed with 0.1 ml of 2 M pH 9 ammonium chloride buffer and 10 mg (3.4×10^{-5} mole) of potassium peroxychromate. The preparation was allowed to warm slowly. A foamy protein precipitate formed which would not redissolve in this buffer or water. It retained a persistent yellow color which could not be washed off. A control of the same buffer and amount of lysozyme with 10 mg (5.1×10^{-5} mole) of potassium chromate was allowed to stand beside the preparation. The control did not precipitate. The protein fraction retained a distinct yellow color after gel filtration chromatography on a 25 cm. Sephadex G-25 column in 5% acetic acid. No further work was done with these protein preparations.

RESULTS AND DISCUSSION

Oxidation of a Protein and Amino Acids by Microwave Generated Singlet Oxygen

At the time these studies were initiated, it was generally assumed that the dye sensitized photooxidation of proteins and amino acids might proceed by a type II, singlet oxygen mechanism (Footé, 1968). The work of Weil (1965), Bellin and Yankus (1968), Jori et al. (1969a, 1969b) and others gave evidence for other mechanisms but they do not seem applicable to the more commonly used oxygen rich aqueous systems, sensitized by methylene blue and rose bengal, where His, Met and Trp are all observed to be oxidized.

In spite of the general assumption that the usual mechanism is singlet oxygen mediated, it was not even known if proteins and amino acids could be oxidized in a dye free system by singlet oxygen. At least one very sketchy preliminary report (Possani et al., 1970) suggested that this was possible. Possani et al. reported that "an effect on hemoglobin" was observed with a system where singlet oxygen was presumably generated in aqueous phase in one chamber by the reaction of hypochlorite and hydrogen peroxide and swept by a nitrogen stream into another compartment which contained the substrate. Little was said about the nature of the changes which took place in the hemoglobin. Hemoglobin does not lend itself to activity assays and no amino acid analysis was reported.

The reports from the laboratory of D. R. Kearns (Merkel et al., 1972; Nilsson et al., 1972; and Nilsson and Kearns, 1973) of the effects on the rate of dye sensitized photooxidations of proteins and amino acids in solutions which either enhance or decrease effective singlet oxygen lifetime gave indirect evidence for its action in these oxidations. The tests consist of running the photooxidations in either deuterium oxide or dilute (typically 0.01 M) azide-water solutions. The lifetime of singlet oxygen is about 20 μ seconds in H_2O . It is less than 0.5 μ seconds in 0.01 M azide. Hence, photooxidations, everything else being equal, should proceed up to 10 times as fast in deuterium oxide and less than one fourth as fast in 0.01 M azide compared to the same reaction run in water. They applied these tests to the methylene blue sensitized photooxidation of His, Met (azide only), Trp, trypsin and alcohol dehydrogenase (both azide and D_2O) with results consistent with a type II singlet oxygen mechanism. Others have since applied their tests to photooxidation of lysozyme sensitized by acridine orange (Schmidt and Rosenkrantz, 1972) and eosin (Kepka and Grossweiner, 1973) with results consistent with singlet oxygen mechanisms. The identity of amino acid products were not reported in these studies.

The goal of my investigation was, then, to determine if dye free singlet oxygen would react with proteins and amino acids and if so, what the identities of the products were. The model systems

selected for this study used singlet oxygen generated by microwave discharge in low pressure oxygen. Lysozyme (chicken egg white) was selected as a model protein because it is readily available, well characterized, stable and amenable to enzymatic activity assays. This last point is important. Other studies by the author (not reported here) involving the dye sensitized photooxidation of myoglobin were inconclusive largely due to lack of a convenient activity assay.

The model amino acids selected for chemical product studies were free Met, N-acetyl and N-glycyltryptophan (NAT and NGT) and N-benzoylhistidine (NBH). NAT and NGT were selected instead of Trp to avoid the complications of melanin formation when Trp is not blocked at the amino group (Benassi, 1967). NBH was selected, instead of His, to provide fluorescent quenching for analytical purposes as suggested by Tomita et al. (1969). The photooxidation products of His do not react with ninhydrin or Pauly's reagent and, thus, are hard to visualize on thin layer chromatograms if a fluorescent quenching "handle" is not provided.

Products of rose bengal and methylene blue sensitized photo-oxidations of the same substrates were prepared for comparison by thin layer chromatography. These comparisons were run with a number of different solvent-absorbent combinations to provide the greatest possible differentiation.

A. Inactivation of lysozyme in solution by oxidation with microwave discharge generated singlet oxygen. When lysozyme was oxidized by microwave discharge generated singlet oxygen in liquid (unbuffered water) solution, (as described in the Methods section), it was totally inactivated in two hours. Samples taken at 0.5, 1.0 and 2.0 hours of oxidation had activities of 100, 50 and 0 percent, respectively, relative to the starting solution. A control, performed to investigate the effect of vigorous low pressure bubbling of oxygen through the enzyme solution had activities of 100 and 68 percent at 1.0 and 2.0 hours, respectively. As the inactivation was observed, and it appears to be specifically dependent on the presence of singlet oxygen, since the control was inactivated only to the extent of 30% when the oxidized material was completely inactive, further basic work with this system was in order. The protein work was set aside and work with amino acids to check the chemical nature of oxidation products of protein constituents was initiated.

The lengthy times of reaction seem to be typical of liquid systems. Several authors had reported oxidation of olefins and nucleic acid components in similar reactors, but they did not usually make any note of the length of time of their reactions. Work that was used as a particular guide for my experimental set up was reported by Gleason, Rosenthal and Pitts (1970). A discussion with Dr. J. W. Peters, of Pitts' laboratory, supported the finding that reactions of

this type are slow and inefficient (Peters, 1972). In a system of this type, singlet oxygen must travel from the discharge zone, be dissolved in the liquid (at a low pressure where solubility is poor) and diffuse to the substrate for reaction before it is quenched. The average lifetime of $^1\Delta_g$ oxygen in water has been determined to be about 2 μ seconds (Merkel and Kearns, 1972). Taken together, the inefficiency of such a system is not too surprising.

During the time that the basic studies with amino acids were being made, Churakova et al. (1973) published their study of the oxidation of lysozyme in a system similar to that described above.

They reported that lysozyme could be inactivated by radio-frequency discharge generated singlet oxygen and that His and Trp disappeared in a manner which paralleled that of methylene blue sensitized photooxidation. They did not report on the identity of the products from the oxidation of His and Trp.

Because this report largely answered the major questions (except for the chemical identity of the amino acid products) about the reactivity of proteins in aqueous dye free singlet oxygen systems, no further work was done here with this system.

B. Oxidation of amino acids by microwave discharge generated singlet oxygen. Table 2 lists the results of thin layer chromatographic comparisons of oxidations of Met, (NBH), (NGT) and (NAT).

Table 2. Thin Layer Chromatographic Comparison of the Products of Photooxidation of Amino Acids with the Products of Oxidation by Microwave Discharge Generated Singlet Oxygen.
R_f (X 100)

Substrate Run Codes	Solvent 1				Solvent 2		Solvent 3				Solvent 4				Solvent 5				Solvent 6				Solvent 7			
	U	MW	RB	MB	MW	RB	U	MW	RB	MB	U	MW	RB	MB	U	MW	RB	MB	U	MW	RB	MB	U	MW	RB	MB
Met																										
06093		31	31		39	37																				
08223	32						47				54							41					56			
09143							72	73			53	54			54	53			41	38			54	55		
Met Prod. (MetSO)																										
06093		10	09		22	20																				
08223		11	12				33	33			39	40						30	29				61	62		
09143							60	58	58		41	45	43		49	47	48		27	27	26		67	68	68	
NBH																										
09143	39	41					74	73										46	45							
NBH Prods		34	35	37			52	55	55									23	26	24						
09143		51	51	51			57	62	59									28	30	30						
NAT																										
06093		56	56		50	50																				
09143	61	58					72	75			45	45						45	43							
NAT Prod.																										
06093		45	46		45	47																				
09143		50	53	51			79	80	81		50	50	50					45	47	47						
											40	40	40													
NGT																										
07283		33	34																							
08223	37						43	42			65	69		42				40				10				
09143	44						72											40				10				
NGT Prod.																										
07283		20	20																							
08223		28	27				26	27			74	75			34	36		33	34			15	16			
09143			35	37						78	77							42	41			16	16			

Solvents: 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
U-untreated starting material, MW-microwave reactor oxidized, RB-Rose Bengal sensitized photo-oxidation, MB-methylene blue sensitized photooxidation

Visualization was discussed in detail in the Methods and Materials section. Briefly, Met and NGT and their products were visualized with ninhydrin. Pauly's reaction was used with NGH. Ehrlich's reaction was used with NGT, NAT and their products. Fluorescent quenching was used to detect NBH, NGT and NAT and their products.

The values obtained on various days with various runs are noted from one run to another. This is typical of results observed using mixed solvents on TLC plates of varying degrees of activation (see Benner et al., p. 391 in Stahl et al., 1965). However, even though there was considerable variation from one run to another, in any given run the R_f values of microwave and dye sensitized photo-oxidation products matched and R_f values were in the same order from one plate to another. The close correspondence of the products run at different times using different solvents and adsorbant systems is good evidence that the products are identical.

The products have been identified in the literature as methionine sulfoxide (MetSO) from Met (Weil et al., 1951; Jori et al., 1968), N'-formyl-N-(acetyl or glycyI)-Kynurenines from NAT and NGT (Benassi et al., 1967; Savage, 1971), and a complex mixture of up to 20 or so products from NBH (Tomita et al., 1969).

Work reported in the section on the dye free singlet oxygen oxidation of Trp shows that the product of the hydrolysis of the product of rose bengal sensitized photooxidation of NGT is identical with

authentic Kyn, supporting the above reports. The preparation of MetSO from Met by dye sensitized photooxidation has been reported by a number of authors and can be considered essentially a standard preparation. Only two products were always observed with NBH. No attempt was made to identify them, other than noting that they were no longer reactive in Pauly's reaction indicating disruption of the imidazole ring.

The apparent identity of the products of dye sensitized photooxidation and microwave generated singlet oxygen oxidation of these amino acids and the ability of dye free singlet oxygen to inactivate a protein provides direct evidence for the mediation of singlet oxygen in the dye sensitized photooxidation of amino acids and proteins. These results clearly show that the products observed from dye sensitized photooxidations can also be produced by a dye free singlet oxygen system. This, taken together with the reports (discussed above) by Nilsson et al. (1972) and Churakova et al. (1973), makes it almost a certainty that dye sensitized photooxidations of proteins sensitized by rose bengal and methylene blue do employ a singlet oxygen mechanism.

Hematoporphyrin Sensitized Photooxidation of Lysozyme: Effects of Singlet Oxygen Enhancement and Inhibition

The hematoporphyrin sensitized photooxidation of lysozyme (chicken egg white) was investigated to confirm and extend the reports of Jori et al. (1969b, 1971). They reported that when a lysozyme

molecule was sensitized by hematoporphyrin in a photooxidation, one of its two methionines was rapidly and cleanly converted to methionine sulfoxide, yielding a product of 53% enzymatic activity. The initial photooxidation took place in about six minutes, giving the one MetSO, but up to 360 minutes did not affect any further photooxidation. No other residue was oxidized, not even the ones usually sensitive to photooxidation. They also reported that, with free amino acids, hematoporphyrin would sensitize the photooxidation of only Met of the usually susceptible ones: His, Trp, Tyr, Cys and Met (provided Trp was blocked at the amino group).

This system was, then, of considerable interest as it offered the possibility of a highly selective way to modify Met in an intact protein to investigate its significance in the molecule. This also seemed, due to its high specificity, to be an excellent candidate for a Type I sensitized photooxidation mechanism involving a direct interaction between the dye molecule and the substrate, avoiding a singlet oxygen mediator which would be expected to be less specific in its action. Both of these aspects were of interest when this investigation was started. Jori et al. (1971) also reported that the interaction of lysozymes with hematoporphyrin caused a shift in the absorption spectrum of the dye. Investigations of this are reported here in a later section.

In a series of preliminary experiments, not reported in detail here, the author was unable to reproduce the reported data of Jori et al. (Extensive reports of this data are not included here as they essentially duplicate the controls in the following reports.) In the preliminary experiments, hematoporphyrin was used to sensitize the photooxidation of lysozyme at pH 6.2 (well within the pH 2-6.5 range reported by Jori et al.) with the loss of 70-90% of the enzymatic activity (vs. the 47% reported). When the amino acid compositions of these extensively photooxidized products were examined, considerable loss of His was noted. The loss of His was notable even in cases where little to no Met was oxidized.

The results obtained by the author were more consistent with what has been observed by others for the dye sensitized photooxidations of proteins and their constituents sensitized by the more common sensitizers methylene blue and rose bengal (cf. Weil et al., 1952, for lysozyme with methylene blue).

It was of interest then to investigate the mechanism of the hematoporphyrin sensitized photooxidation of lysozyme by the most definitive methods available. This proved to be the test systems of Nilsson, Merkel and Kearns (1972; Nilsson and Kearns, 1973), discussed in detail in the preceding section, utilizing deuterium oxide and azide photooxidation media to respectively enhance or inhibit singlet oxygen oxidations.

The first experiment reported here examined the difference in rate of hematoporphyrin sensitized photooxidation at pH 6.2 in the presence and absence of 10^{-2} M azide. The enzymatic activity was used as an index of the extent of reaction. The second experiment reports the effect on this system of 10^{-3} M azide and 10^{-3} M DABCO (1,4-diazabicyclo[2.2.2]octane).

Subsequent experiments report the effect of running the sensitized photooxidations in deuterium oxide and 0.02 M azide at pHs of 4.7 and 6.2.

A. Figure 8 shows the results of the first experiment mentioned above. The specific experimental conditions are noted in the Methods section. Briefly, 114 μ M lysozyme was photooxidized with 257 μ M hematoporphyrin in the presence and absence (control) of 10^{-2} M azide. Samples were taken for enzymatic activity analysis at various times (0, 1, 2, 4, 8, 16 and 32 minutes) to determine the extent of reaction. The ordinate of the graph is percent specific activity relative to the original preparation as determined from the Lowry values for protein concentrations.

This determination clearly shows the inhibition by azide of photooxidation sensitized by hematoporphyrin. The inhibition shown here is not as dramatic as that reported by Nilsson and Kearns (1973) for the dye sensitized photooxidation of trypsin and alcohol

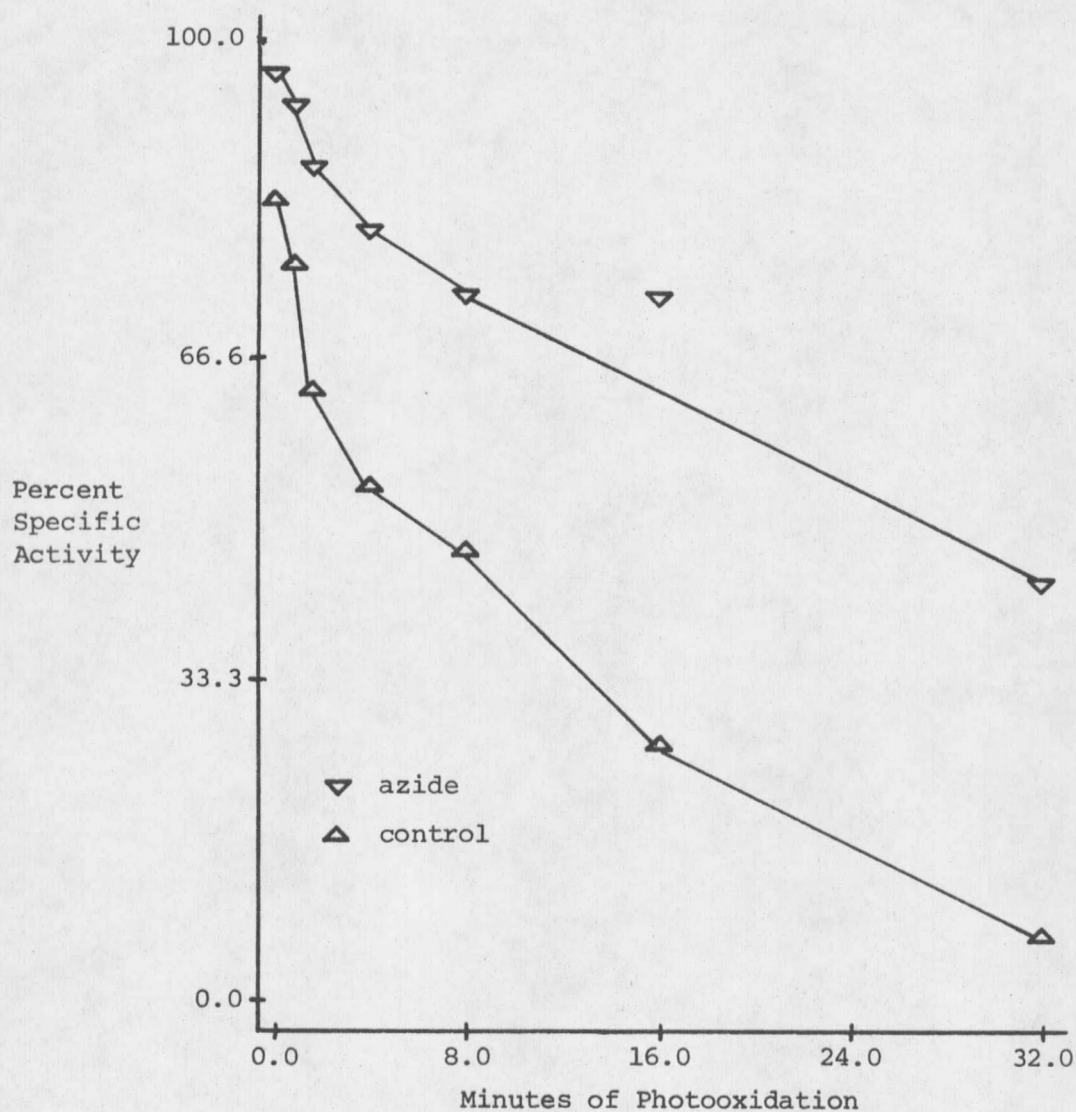


Figure 8. Comparison of azide inhibited and control photooxidations in hematoporphyrin sensitized inactivations of lysozyme in 0.01 M pH 6.2 phosphate buffer. Lysozyme 114 μ M, dye 257 μ M, azide 0.01 M.

dehydrogenase. Using methylene blue as a sensitizer, they reported almost total protection with 10^{-2} M azide. They point out that the lifetime of singlet oxygen in 10^{-2} M azide is less than 0.5 μ seconds vs. the 2 μ seconds observed in water. The results shown here indicate a singlet oxygen mediated reaction.

B. Figure 9 shows the results of the photooxidation in the presence of 10^{-3} M azide and 10^{-3} M DABCO. The selection of these concentrations of inhibitors was due to an error on the part of the author. The azide concentration is a tenth that proposed by Nilsson and Kearns (1972) and which was used to good effect in the preceding experiment. Anderson et al. (1974) report that DABCO is not an effective quencher of singlet oxygen in aqueous systems at 0.05 M, which is 50 times what was used here. The data are included here not as a comparison, as the inhibitor levels are too low to inhibit effectively, but as a demonstration that the hematoporphyrin sensitized photooxidation is reproducible and to provide a basis for the amino acid analysis data. Note that the enzymatic activity values shown for each data point are relative to the value of the first point on each curve. The curves are normalized in this manner to make comparisons clearer. Subsequent data will be shown in this manner.

One of the main goals of this experiment was to verify the report by Jori et al (1969b) that photooxidation sensitized by

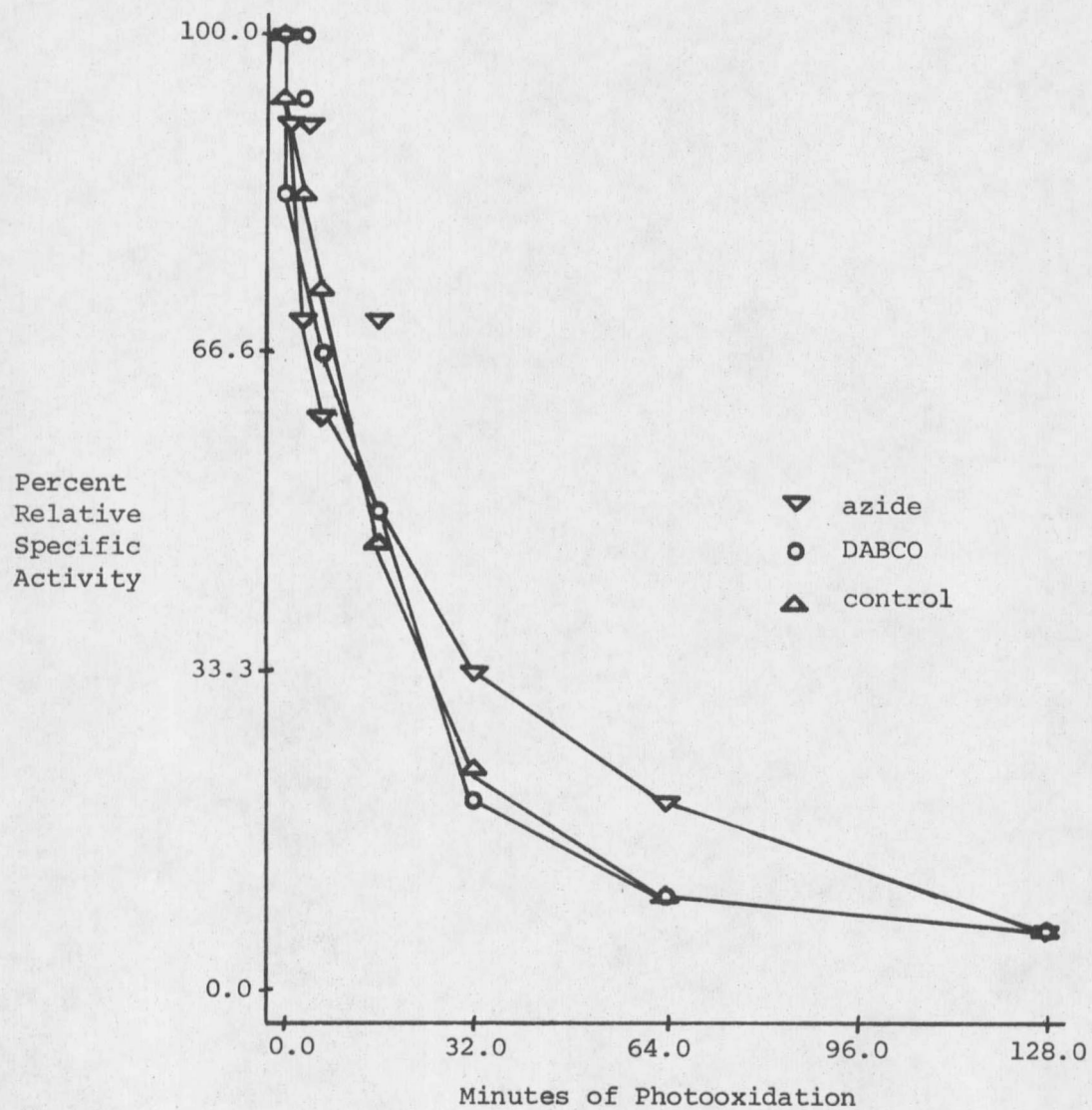


Figure 9. Photooxidation of lysozyme sensitized by hematoporphyrin in 0.1 pH 6.2 buffer. Determination of the effects of low concentrations of the singlet oxygen quenchers azide and DABCO on the rate of inactivation.

hematoporphyrin in the pH range from 2.5-6.5 oxidizes only methionine. Table 3 shows the results of the amino acid analysis of the final photooxidized products. It had been hoped that this experiment would say something about the effect that azide has on the specificity of reaction regarding products. Due to the low level of azide, and its resulting small protection, the azide values can probably be considered essentially duplicate analyses of the control.

The surprising aspect of this data was that, contrary to Jori's reports, there was little apparent damage to the Met, almost complete destruction of the His and extensive damage to the Trp, producing Kyn. This clearly shows the destruction of the His and Trp. In previous determinations of methionine sulfoxide run by 3.3 N sodium hydroxide basic hydrolyses, fairly close agreement was observed for the formation of the sulfoxide and loss of Met. The sulfoxide could be observed in the chart outputs in this case, but the amounts were insignificant, being less than 10% of the Met peaks on the same chromatograms. The lack of effect for the DABCO is consistent with what is known of the mechanism of quenching by this molecule. Young et al. (1973) report evidence that the quenching of singlet oxygen by amines, especially tertiary amines, proceeds by a charge transfer mechanism, ($^1\text{O}_2 + :\text{NR}_3 \rightarrow \text{O}_2^{\bullet -} + ^+\text{NR}_3 \rightarrow ^3\text{O}_2 + :\text{NR}_3$). This would not be expected to be very effective at a pH of 6.2 where tertiary amines like DABCO will be protonated. The nitrogen forms a positive charge, if the

Table 3. Amino acid analysis of lysozyme photooxidized in presence of hematoporphyrin at pH 6.2 in 0.1 M phosphate buffers in the presence and absence of negligible amounts of sodium azide.

Amino Acid Residue	788-74	790-74	Standard ¹
His residues	less than 0.1	less than 0.1	1.0
Met	2.2	2.0	2.0
Trp	2.9	2.9	6.0
Kyn	0.6	1.3	0.0
CysH	5.0	5.7	8.0
Tyr	2.8	2.9	3.0
Lys	5.0	5.3	6.0
Arg	10.4	11.4	11.0
Asp	21.5	24.4	21.0
Thr	6.7	7.5	7.0
Ser	9.8	10.8	10.0
Glu	4.9	5.5	5.0
Pro	1.8	2.1	2.0
Gly	12.6	13.5	12.0
Ala	12.2	13.8	12.0
Val	4.0	4.1	6.0
Ile	4.5	4.5	6.0
Leu	7.4	7.8	8.0
Phe	2.9	2.6	3.0
Enzymatic Activity	7%	8%	

The results shown are for methanesulfonic acid hydrolyzates. The parallel basic hydrolyzates were not computed when visual examination of the data showed negligible amounts of methionine sulfoxide.

788-74 control

790-74 photooxidized in presence of 10^{-3} M azide

¹Standard values for the amino acid content of lysozyme taken from Atlas of Protein Structure 1966, ed. R.V. Eck and M.O. Dayhoff, National Biomedical Research Foundation.

Refer to Table for typical values for unoxidized lysozyme hydrolyzed by the same method.

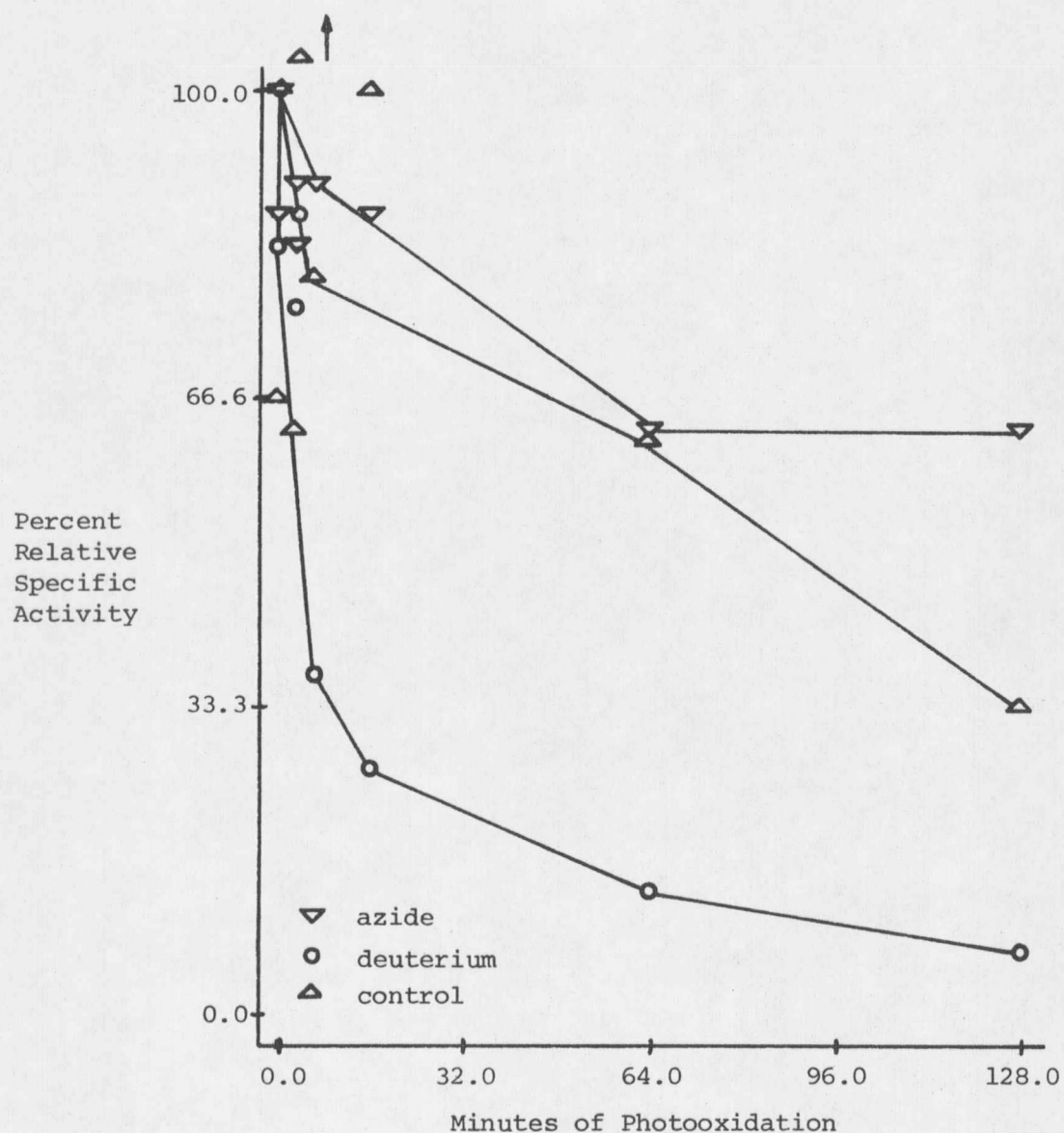


Figure 10. Comparison of the azide inhibition and deuterium enhancement of hematoporphyrin sensitized photooxidation of lysozyme in 0.1 M pH 4.7 acetate buffers. Lysozyme 103 μ M, dye 257 μ M, azide 0.01 M, deuterium greater than 99 atom %.

mechanism is correct, which should be discouraged if a net positive charge is already there.

C. Figure 10 compares the results obtained from hematoporphyrin sensitized photooxidation of lysozyme in deuterium oxide or azide media at pH 4.7. The conditions were: lysozyme 10^{-3} M, hematoporphyrin 2.5×10^{-5} M, azide 10^{-2} M. The scatter of the data, particularly for the control--water-acetate buffer only--limits the usefulness of this result, but the trends are clear. Deuterium oxide medium enhances the sensitized photooxidation process while azide inhibits it. A somewhat less vigorous reaction in the control is noted here at pH 4.7 than in the previous experiment at pH 6.2. Although reaction conditions are sufficiently different, direct comparison is difficult; the lower reactivity is consistent with what has been reported by a number of authors for the pH-dye sensitized photooxidation activity profile (Weil et al., 1952; Sluyterman, 1962).

D. Figure 11 compares the results of inactivation of lysozyme in azide and deuterium systems at pH 4.7 while Figure 12 shows the same comparison at pH 6.2. Table 4 lists the individual data points for both. The results shown for the phosphate pH 6.2 systems are fairly consistent with what has been seen in the previous experiments in this system. The acetate pH 4.7 system results were somewhat surprising. The results shown here more closely resemble those of

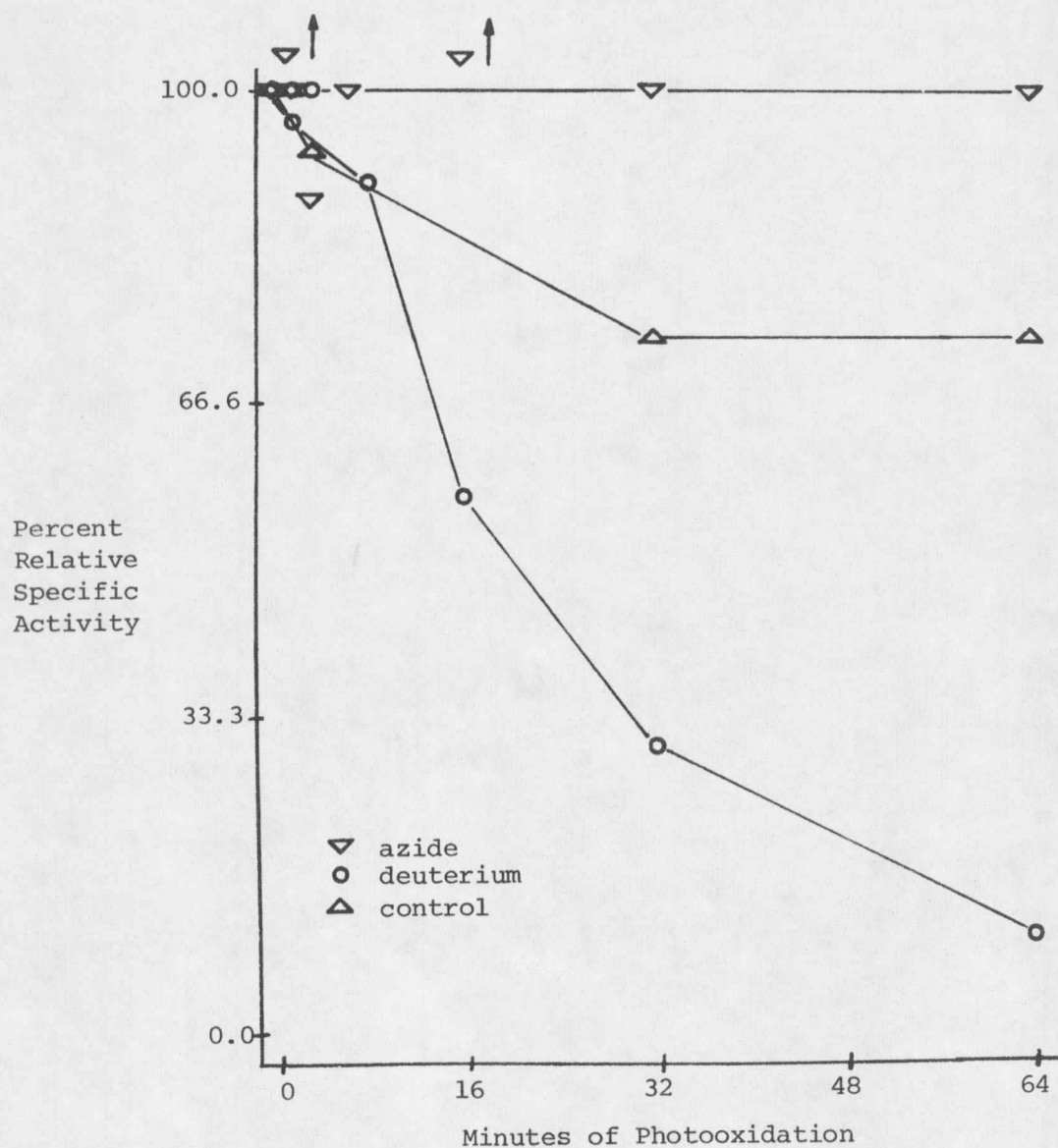


Figure 11. Comparison of the azide inhibition and deuterium enhancement of hematoporphyrin sensitized photooxidation of lysozyme in 0.1 M pH 4.7 acetate buffers. Lysozyme 105 μM , dye 205 μM , azide 0.02 M, deuterium oxide greater than 99 atom % ^2D .

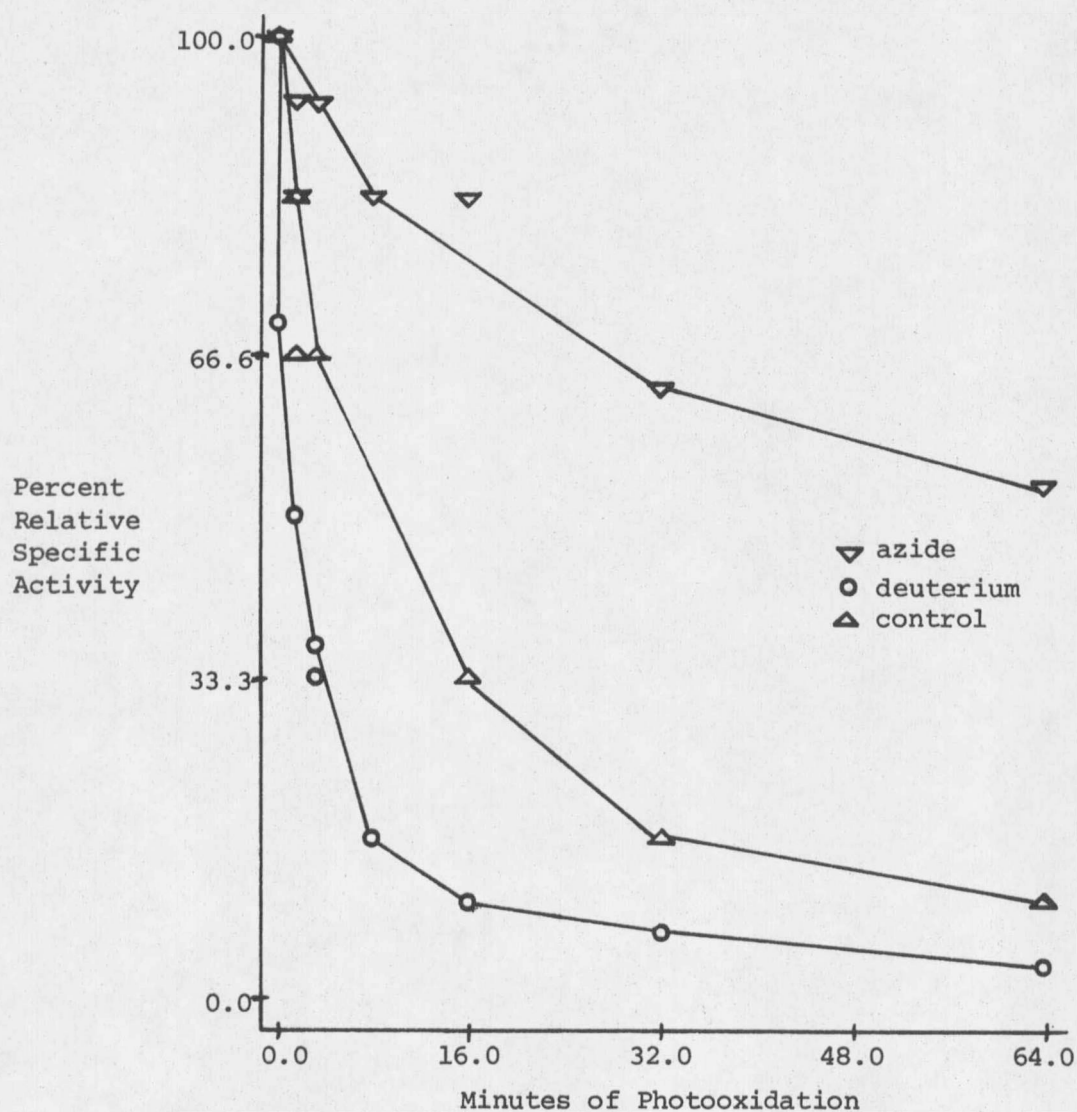


Figure 12 Comparison of the azide inhibition and deuterium enhancement of hematoporphyrin sensitized photooxidation of lysozyme in 0.1 M pH 6.2 phosphate buffers. Lysozyme 105 μ M, dye 205 μ M, azide 0.02 M, deuterium oxide greater than 99 atom % deuterium.

Table 4. Relative percent specific activities and percent specific activities for the determinations detailed in Figures 11 & 12

Time of Sample (min.)	pH 4.7 Control	Acetate Azide	Buffers Deuterium	pH 6.2 Control	Phosphate Azide	Buffers Deuterium
0	100 80.5	100 80.3	100 93.5	100 98.	100 95.5	100 95.5
1	100 80.5	122 98.	96 90.	82 80.5	85 91.	64 61.
2	-- --	99 79.5	102 95.5	68 66.3	84 80.	49 47.
4	93 75.	86 69.	102 95.5	68 66.3	93 88.	34 32.
8	-- --	101 81.5	90 84.5	39 38.	84 80.	17 16.
16	-- --	111 89.	58 54.5	32 31.	84 80.	11 10.
32	72 58.	100 80.5	30 28.5	16 15.5	63 60.	7.8 7.5
64	74 60.	101 81.5	9.6 9.	9.5 9.3	53 51	4.2 4.0
0.5			100 93.5			71 67
3			102 95.5			37 36

The values for 0.5 and 3 minutes for the deuterium experiments were not plotted in the figures.

Nilsson and Kearns (1973) where azide nearly completely inhibits the sensitized photooxidation.

If it were not for the nearly absolute inhibition observed in the acetate buffer system with 0.02 M azide, it would be tempting to interpret the results shown here as a shift from a Type I mechanism, of dye bound as suggested by Jori (1971), to a Type II mechanism in the presence of deuterium stimulation. The possibility that azide quenches the triplet state of the sensitizer, directly blocking both Type I and Type II mechanisms, can't be ruled out. Work by Hasty et al. (1972) and Foote et al. (1972) suggest that this is not very likely as studies with other sensitizers show poor competition of azide with oxygen for reaction with dye triplet. Jori's suggestion that the dye is bound, might, however, explain the differences observed in this experiment and the previous one with a higher dye:enzyme ratio. That some sort of interaction takes place between the dye and the protein is indisputable, as in solutions of greater than about 50 μM lysozyme, eventually the dye and, apparently, most of the protein will precipitate giving colorless solutions. This is at both pH 4.7 and 6.2. In more dilute solutions, e.g., 10 μM , the solution is stable and will remain pink indefinitely with no visible precipitate. The precipitation is a function of both the dye and the protein as either alone is stable at pH 6.2 at up to 1000 μM concentrations. At 1000 μM , the combination precipitates rapidly. One might speculate

that the slightly higher dye:protein molar ratio in the preceding experiments 2.6:1 vs. 2:1, might permit enough unbound dye to serve as a more efficient sensitizer for the readily susceptible residues of the protein. The bound dye may sensitize, but due to being bound, may do so poorly because of either steric considerations (the oxygen can't get to the dye) or electronic ones (the bound dye triplet either does not form or is quenched). The singlet oxygen may not have access to susceptible residues as easily as it could if generated free of the protein. The same suggestion can be applied to the differences between the pH 4.7 and pH 6.2 results. Possibly, there is more free dye available to sensitize oxidation in the 6.2 case. It is normal to see higher sensitized photooxidation sensitivity at higher pH. The difference here is 1.5 units in the region where the His imidazole loses its proton. This may be a more likely reason for the difference.

Whatever the fine points of the reaction may be, the conclusion, from the results shown here, that the main photooxidation process is singlet oxygen mediated seems inescapable.

The general observations that can be made from the amino acid analyses shown in Table 5 are as follows:

1. In both systems, acetate and phosphate, oxidation of His is very active in deuterium oxide. The loss of His seems to parallel loss of activity more than other residues. This is typical

Table 5a. Amino acid analyses of lysozyme photooxidized in the presence of hematoporphyrin with deuterium oxide or azide present: methanesulfonic acid hydrolyzates

Amino Acid Analyzer Run Code:						
Residues	Reported Standard	815-74	816-74	817-74	813-74	818-74
His	1	trace	0.7	0.8	trace	0.1
Met	2	1.8	1.9	1.7	1.1	1.7
MetSO	0	0.2	0.2	0.2	1.0	0.5
Cys	8	5.1	5.2	5.2	5.5	5.1
CySO ₃ H	0	trace	trace	0	0.2	0
Trp	6	3.4	5.0	4.1	1.8	4.5
Kyn	0	1.0	0.5	0.1	1.3	0.3
Tyr	3	2.9	3.0	2.9	3.0	2.9
Asp	21	22.0	22.4	21.8	23.3	21.4
Thr	7	7.0	7.2	7.0	7.5	6.9
Ser	10	9.8	9.9	9.5	10.3	9.4
Glu	5	4.9	5.1	4.9	5.2	4.8
Pro	2	2.0	2.1	2.1	1.9	2.1
Gly	12	12.5	12.8	12.4	13.2	12.1
Ala	12	12.2	12.7	12.2	13.0	12.0
Val	6	3.8	3.6	3.8	3.8	7.3
Leu	8	7.3	7.4	7.3	7.8	4.3
Ile	6	4.1	4.3	4.3	4.5	2.7
Phe	13	2.6	2.6	2.7	2.8	2.7
Lys	6	5.1	5.3	5.6	5.7	5.1
Arg	11	9.8	10.2	10.8	10.6	10.6
Ammonia	16	23.1	21.0	21.9	24.5	23.1
Enzymatic Activity	100	9	74	100	4	53

815-74 - pH 6.2 acetate-D₂O

816-74 - pH 4.7 acetate control

817-74 - pH 4.7 acetate azide

813-74 - pH 6.2 phosphate-D₂O

818-74 - pH 6.2 phosphate azide

From Table 3 - pH 6.2 water 10% act. < 0.1 His, 2.2 Met, 2.9 Trp.
0.6 Kyn

Table 5b. Amino acid analyses of lysozyme photooxidized in the presence of hematoporphyrin with deuterium oxide or azide present: basic hydrolyzates

Residues	Reported Standard	Amino Acid Analyzer Run Codes:			
		820-74	819-74	821-74	814-74
Met	2	2.1	2.0	2.0	1.6
MetSO	0	0	0.3	0.2	0.4
Asp	21	15.8	9.3	18.6	17.3
Thr	7	0.7	0.7	0.1	0.8
Ser	10	3.4	3.7	2.4	3.9
Glu	5	1.4	1.4	4.5	1.6
Pro	2	0.7	0.4	1.1	0.8
Gly	12	13.1	10.7	18.8	17.1
Ala	12	16.4	13.4	18.1	18.3
Cys	8	0.1	0	0.2	0
Val	6	2.7	2.6	2.6	3.0
Ile	6	2.6	2.6	2.5	2.8
Leu	8	8.1	8.3	7.5	8.6
Tyr	3	3.0	2.8	2.9	3.0
Phe	3	2.3	2.1	2.3	2.6
Enzymatic Activity	100%	4	74	100	9

814-74 - Phosphate-Deuterium oxide
 819-74 - Acetate-Control
 820-74 - Acetate-Deuterium oxide
 821-74 - Acetate-Azide

of what is observed with the methylene blue sensitized photooxidation of lysozyme (Weil et al., 1952).

2. In the controls and azide systems, the higher pH phosphate system favors oxidation of the His. In fact, it seems well protected at pH 4.7 in water and azide.

3. Met seems to be more susceptible at pH 6.2 in deuterium oxide than at pH 4.7. This may be due to unfolding when the His is oxidized.

4. Not much can be said about the oxidation of Cys. There does seem to be a slightly greater loss at pH 6.2, but this is probably not significant.

5. The amount of Kyn produced and the amount of Trp missing has yet to balance in a hematoporphyrin sensitized oxidation of lysozyme. This is somewhat contrary to the quantitative conversions and recoveries reported by Galiozzo et al. (1968) for the proflavin sensitized photooxidation of lysozyme and the work reported here on non dye singlet oxygen oxidation of Trp. This may be more in line with the work of others where they observe other products (Asquith and Rivett, 1971) and not a quantitative Kyn production.

6. Tyr was not destroyed under these conditions. Actually, demonstrations of Tyr destruction by sensitized photooxidation of an intact protein are rare under pH 9.

7. The identity of the residues destroyed is apparently not affected by the presence of either azide or deuterium. The only obvious effect that they have is one the overall rate of oxidation. The pH appears to be the only factor which has a definite effect on which residues are oxidized.

A recent report by Walrant and Santus (1974) discusses the quantum yields of the photooxidation of various amino acids, particularly Trp, His and Tyr, both free and in small peptides, sensitized by N-formyl-Kyn. Irradiations were with light of 320 nm, and longer, wavelengths, avoiding the Trp and Tyr absorbances at 280 nm. They used the deuterium oxide and azide tests. They found that, at pH 7.6, the His seems to be oxidized solely by singlet oxygen. It has relative quantum yields of 5.8, 29 and 0 for water, D₂O and azide (10^{-2} M), respectively. The corresponding values for Trp are 14, 49 and 6.4. The values in water for Trp in bovine carbonic anhydrase, Trp-Tyr peptide and N-methyl-Trp were 3.8, 5.5 and 8.7, respectively. This together with some other data was interpreted as showing a combination of mechanisms with Trp. They suggested that the major pathway in water was singlet oxygen, with a component, possibly due to

superoxide ion (O_2^-), which becomes evident in azide solutions. Unfortunately, they did not report on the effect of the deuterium/azide test with the peptide, protein or N-blocked Trp to see if mixed mechanisms are important there.

This interpretation would be consistent with the loss of two Trp but only 0.2 His in the acetate azide results reported here. Nothing in their report bears directly on conditions at pH 4.7 as they worked at pH 5.7 and higher.

Kepka and Grossweiner (1973) reported on a system of photo-oxidation of lysozyme sensitized by eosin which has some features similar to what was reported here. They presented good evidence for the binding of one mole of eosin per lysozyme, at pHs of 4-11, close enough to Trp-108 to quench its fluorescence. At low pHs, 4-6, they report that the quantum efficiency roughly parallels the percentage of dye bound, but that surprisingly, the Trp is somewhat protected. When they go from water to deuterium oxide, the Trp destruction is the same or less, even though overall inactivation quantum efficiency is 5-10 times greater suggesting an "internal singlet oxygen" between the dye and the Trp which is relatively less affected by the presence of D_2O on azide.

The results of the experiments reported here are completely consistent with the hematoporphyrin sensitized photooxidation of lysozyme proceeding by a singlet oxygen Type II mechanism. The

observations of enzymatic inactivation, in the case of the reactions run in simple buffers as well as those performed in deuterium oxide and azide solutions, and amino acid losses fail to support the observations reported by Jori et al. (1969b, 1971). These results are similar to what has been observed for inactivations sensitized by methylene blue and other dyes and simply can not support a highly selective mechanism.

Dry State Oxidation of Amino Acids and Proteins by Microwave Generated Singlet Oxygen

A. Thin layer oxidation of amino acids. The oxidation of amino acids, and other substrates, directly on a thin layer chromatography sheet was originally attempted to save some time and effort in the study of the interactions of singlet oxygen generated by the microwave reactor. Previous studies, reported in the section on "Oxidation of a Protein and Amino Acids by Microwave Generated Singlet Oxygen" showed that while singlet oxygen generated in this system would oxidize amino acids, the reaction is slow and inefficient. This is presumably due to the difficulty of getting the singlet oxygen to the substrate before it is quenched by the water solution.

A preliminary check showed the operation of this system is rapid and convenient and that oxidation of Met on silica gel thin layer sheets occurs easily.

1. When 10 μ g of Met was exposed to singlet oxygen on Bakerflex 1B silica gel TLC sheet for 15 minutes, as described, developed with the BAW eluent and visualized with ninhydrin, spots, having R_f 's of 0.42, 0.23 and 0.13, appeared. A control of 10 μ g on the same type TLC sheet, but not exposed, showed a single spot at R_f 0.40. Visual estimation of the size and density of the spots on the oxidized strip showed over half of the total Met was in the R_f 0.13 spot. The 0.23 spot was minor, estimated at less than 20% of the total while the 0.42 spot, presumably unoxidized Met, approximated 30%. These R_f 's compare with other runs on Met and its products of photooxidation, reported in Table 2 (developed with BAW) of 0.32 and 0.11. A comparison of material oxidized in the presence of rose bengal had R_f values of 0.46, 0.28 and 0.18. The 0.13 spot correlates with methionine sulfoxide (MetSO) while the minor 0.28 spot seems to be methionine sulfone (MetSO₂), although this was never unequivocally established. This spot was not observed unless photooxidation of Met was continued to nearly completion so its appearance here when a sizable portion of the Met had not reacted was puzzling.

2. When this technique was used with His (not NBH), Met, NGT, Ala and Cys₂, the following R_f 's were observed with the oxidized materials (BAW and ninhydrin): His 0.21 (weak spot), 0.10; Met 0.40, 0.21, 0.11; NGT 0.44, 0.36, 0.27; Ala 0.23; Cys₂ 0.16. Control values

were: His 0.10; Met 0.38; NGT 0.37; Ala 0.23; Cys₂ 0.21. Comparison values from photooxidized materials are (from Table 2): His not observed; Met 0.46, 0.18; NGT 0.46, 0.36; Ala not done; Cys₂ not done. The results for the Met and NGT fairly well correspond to the photooxidized results. The lack of obvious reaction with Ala is consistent with its reported dye sensitized reactions and what was observed in the section on non dye oxidation of Trp here. While several solvents have been tried, no good system for TLC of Cys₂ and its photooxidation products were developed here. Not a great deal of effort was put into this search. What was observed was of such poor quality (long tailing and no differentiation) that no other mention is made of it in this report. BAW gives a badly streaked Cys₂ spot. No product spot had been previously observed with His, which is why NBH was generally used in these studies. The product spot that was observed here was very faint, while the parent spot showed considerable diminution compared to the control.

From this, it was concluded that the reaction of amino acids in this system was similar to dye sensitized photooxidations or microwave discharge flow system liquid oxidations. This system seems to be more reactive than dye sensitized systems and much more reactive than the liquid flow-discharge reactions.

3. A few surprising results were obtained from the attempts as described to optimize the reactor conditions. Very little difference could be detected between any pressure or power setting or length of oxidation in the nature of the results with Met. Three spots were always observed. The ones with R_f 's of 40-43 (parent Met) and 12-15 (MetSO) were approximately equal in density. While the one at 23-26 was faint, perhaps 10% of the others. The main difference between the extent of oxidation noted in this series and the previous two proved to be the brand of the silica gel TLC sheet. Bakerflex 1B gives better conversions with 10 μ g spots than EK 13191. The NBH results were puzzling. Tomita et al. (1969) reported up to 20 products from dye sensitized photooxidation of NBH. Only two product spots were observed on TLC's reported in Table 2 from both solution microwave generated singlet oxygen and dye sensitized oxidations. Typical BAW R_f 's for these were 0.34 and 0.51 with the parent spot at about 0.39. In short, dry exposures (15 min), NBH gave three spots of R_f 's 0.39-0.40, 0.58-0.62 and 0.71-0.74. At one hour, two spots were observed at 0.28 and 0.39. The 0.39-0.40 spots are starting material, but the others don't correlate well at all, although the 0.28 might correspond to the 0.34 dye product. This is the only instance of non correspondence between dye sensitized photooxidations and the microwave system, wet or dry observed in this work. In view of the complicated nature of the NBH system, I am unable to ascertain

its significance. Tomita reported that the nature of the products observed changed with length of reaction time, since some which formed initially went on to other products.

The NGT gave two spots, the parent at 0.39-0.43 with a product at 0.30-0.31. The NGT results were visualized with both ninhydrin and Ehrlich's reagent. The product spot gave a yellow reaction typical of Kyn and its derivatives. These values compare with ones of 0.39-0.44 and 0.29-0.30 from earlier work. Nothing useful was learned from His or Tyr. His showed only the parent spot and Tyr showed a long streak from the origin to about R_f 0.4, as did an unoxidized control.

4. When a thin film of Met ($40 \mu\text{g}/\text{cm}^2$) on glass was exposed in the reactor for one hour and then chromatographed, as described, apparently nothing had happened. One spot was observed in the chromatogram at R_f 0.39.

5. The same results were observed when the Met was exposed on a cellulose acetate strip.

6. No useful results were obtained from the oxidation of Met on alumin TLC sheets. None of the eluents used would differentiate between Met and standard photooxidation produced MetSO. No

data on TLC of amino acids on alumina were available in any of the handbooks, a fact for which I suspect there is a good reason.

7. When oxidation of Met was attempted on MN-300 microcrystalline cellulose TLC sheets, nothing happened. The oxidized material matched the starting material at R_f 0.50 on the BAW developed sheet and 0.69-0.71 on the propanol-butanone-HCl sheet. Photooxidation produced MetSO ran at 0.34 and 0.37, respectively.

8. When Met was exposed before and after being chromatographed on the silica gel TLC sheet, as described, then rechromatographed at right angles, complete conversion was noted on the second exposure. The same thing done with MN-300 cellulose showed some small conversion on the second exposure. From this, it was concluded that the Met has to be spread out enough to be in intimate contact with silica gel surface and available to the gas for the oxidation to take place. In other runs, where incomplete conversion was noted, the Met was probably "piled up" with regard to the surface. The fact that Met will be oxidized on silica gel and not on cellulose or glass suggests that a combination of surface area and possibly catalytic effects may be operating.

9. It was felt that possibly the oxidation could be induced on an acid surface, since silica gel is acid. Acidified

carboxymethyl cellulose TLC plates were used. There was no difference between the oxidized material and the starting material, but both were much different than standard MetSO. The R_f 's were about 0.50 for Met and the exposed material and 0.30 for MetSO.

10. When oxidation was attempted on Whatman Chromatography paper, no results were obtained, as on all the other celluloses.

11. and 12. When CO_2 or a 10% O_2 -90% He mixture was used in the discharge reactor, no difference could be observed in the results. A violanthrone spot with CO_2 gave a rather faint glow, indicating a poor yield of singlet oxygen. This result, coupled with the observation that pressure and power settings, some known to be poor for singlet oxygen production, and time of exposure have little effect on the yield of MetSO leads to the conclusion that only the Met in a certain (unspecified) relationship to the silica gel can react while the rest can not or will do so slowly. The material that can react, will react, if any singlet oxygen at all is available. One unfortunate aspect of the equipment available for this work was the lack of a low pressure gas flow meter to permit measurement of oxygen mass flows and some method of absolute calibration of the concentration of singlet oxygen. I might point out here that approximately 1.5 μl (1.5 mm^3) of STP oxygen are necessary to react with 10 μg of Met. Very rough calibrations of the flow rate in this reactor at

three torr gave amounts on the order of seven ml a second STP (0.3 m mole/sec). Scheffer and Ouchi (1970) report that a similar reactor, under similar operating conditions, has been estimated to produce about 6% delta singlet. These operating parameters should provide a large excess of singlet oxygen for reaction. The only time that it appeared as though the system might be short on usable singlet oxygen was in work where three g of silica gel was present in the reactor (cf. sections B-8 & 10 and C following for further discussion of this).

13. Dry state dye sensitized photooxidation of Met seems to be a simple, rapid and convenient technique. Simply placing the substrate (Met) on the TLC sheet and overlaying it with a small amount of sensitizer, then exposing it to light gave rapid conversion of about the same proportion of the Met to MetSO as was observed in the equivalent use of the microwave reactor. There are a couple of obvious advantages to this technique:

a. It's fast and convenient; b. it permits use of dye sensitized photooxidation, in cases where dye and substrate have solvent incompatibilities (i.e., most sensitizing dyes are soluble in solvents of the acetone-ethanol-water ranges, whereas it is occasionally of interest to photooxidize a large hydrocarbon which is not soluble in any of those. The usual way around this is a complex solvent mixture, often based on pyridine).

This type of sensitized photooxidation proved to be very sensitive to traces of dye. Controls run to test the effect of light alone showed no reaction. If even the slightest trace of rose bengal had contaminated the applying needle, a sizable conversion was seen.

14. NGT was exposed in the reactor on the TLC silica gel sheet and chromatographed concurrently with the photooxidized NGT material described in the section on non dye oxidation of Trp giving the following R_f : BAW-0.46, 0.36, MAC-0.61, 0.57, 0.47. Those matched the photooxidized products supporting the contention that there is no essential difference in the chemistry of the reaction between wet and dry microwave generated singlet oxidations.

I wish I could claim priority for this type of reaction, both microwave generated and dye sensitized, on silica gel but unfortunately I can not. These systems were developed independently, but when I knew what to look for, I discovered that Scheffer and Ouchi had used a similar reactor to oxidize some "model" olefins (e.g., 1,3-diphenylisobenzofuran, tetracyclone, etc.), usually used to test for singlet oxygen reactivity, while adsorbed on silica gel or microcrystalline cellulose. They did not observe the difference between silica gel and cellulose that I did. More significant in this study is the amino acid and protein work. They also made a brief mention of use of dye sensitized photooxidation on TLC sheets or in bulk, but

did not report any results. They noted that this system of sensitized photooxidation on silica gel was similar to Kautsky's classic proof of a gaseous diffusible agent in dye sensitized photooxidation. Just recently, a report by Nilsson and Kearns (1974) discussed a variation of this idea. They adsorbed rose bengal or methylene blue on silica gel, which could then be added to solutions of substrate in nonpolar solvents or mixed with substrate adsorbed on other grains of silica gel for photooxidation. They reported on the photooxidation of 1,3-diphenylisobenzofuran and Trp by this technique. (No product isolation for Trp was reported. They used a spectral technique similar to the one used here in the section on non dye singlet oxygen oxidation of Trp.) To finish this discussion, I would like to quote from their paper.

"We have explored the use of other types of supports with rose bengal (alumina, celite, powdered cellulose) but all were much less effective than silica gel, and this is probably due to their much smaller surface area (Isbell and Sawyer, 1969)."

B. Dry state microwave generated singlet oxygen oxidation of proteins

1. Directly oxidizing lysozyme in free, lypholyzed masses by microwave generated singlet oxygen doesn't seem very effective. The specific activities of material exposed for 1, 5, 10 and 22 minutes to the direct singlet oxygen stream were 102, 85, 86 and 84.

percent of the starting values, respectively. The method of handling was such that small losses of the lypholyzed material when starting the reactor were possible, which might account for the 15% losses of activity as no direct determination was made of the concentration of the analytical solutions.

2. The results of extending direct oxidation of unsupported lysozyme up to six hours were little different than with the shorter times of the previous series. Material exposed for 1, 2, and 6 hours had specific activities of 97, 99 and 81 percent, respectively. In this series, determinations were made of solution concentrations so the 20% loss at six hours is probably real. In any case, the reaction is certainly not spectacular in its vigor.

3. Cellulose was chosen for use as a support-dispersant-absorbant following the method of Scheffer and Ouchi (1970). They reported the use of silica gel and microcrystalline cellulose as absorbants (or adsorbants) in oxidations of this type. At the time that this determination was made, no microcrystalline cellulose was available and silica gel was considered to be excessively harsh for use with enzymes. Fibrous chromatographic cellulose was chosen primarily for availability. The specific activities of material exposed to the excited oxygen stream for 70 minutes and the control material were 63 and 68 percent, of that of the starting material, respectively.

The protein concentrations were determined only by the 280 nm absorbance. It is conceivable that the apparently lowered activity for both samples was due to nonenzymatic material washed from the support being calculated as though it was lysozyme, even though the support was thoroughly prewashed. Other than non-specific effects of absorption and handling, no other reasons can be offered for the decrease in control activity. The net conclusion from these was that little specific oxidation was observed. This is in accord with the results observed with the attempted oxidation of methionine on chromatographic paper, which could be more or less equivalent both chemically and physically to the powdered cellulose used here.

4. Microcrystalline cellulose was obtained and used as a support for the oxidation of lysozyme to follow more closely the conditions of Scheffer and Ouchi. It was hoped that this material would provide a higher surface area for the enzyme to be absorbed on giving good access of the oxygen stream to all protein molecules. Two different techniques were used. The enzyme was frozen in excess water solution with the absorbant, lyophilized and exposed. Because the enzyme does not seem to interact strongly with the microcrystalline cellulose (e.g., it washes off with mild buffer washes), the second technique dried the solution on to the absorbant under vacuum to attempt to insure that it would stick. More than 85% of the enzyme was

recovered from both preparations as determined by the 280 nm absorbance. The material frozen and lyophilized and its control, identical in treatment except for the 40 minute singlet oxygen exposure, both had specific activities of 97 percent. The material vacuum dried on from a liquid and exposed for an hour and its control had specific activities of 79 and 101 percent, respectively.

This last value may represent actual specific oxidation, however, it is of such limited extent that the only conclusion that can be drawn is that lysozyme is not very sensitive to singlet oxygen under these conditions.

5. Since the gentle cellulose absorbants did not permit oxidation of lysozyme in these systems, which paralleled the earlier work with Met on thin layer absorbants, silica gel was tried as a support. The lysozyme was desorbed with 80% acetic acid. The acetic acid solutions were assayed directly. This first experiment with silica gel as a support was inconclusive as no enzymatic activity could be detected in the control. Consideration of the conditions of the experiment suggested that this might be due to any or all of the following reasons: a. The support inactivates the enzyme by denaturing it with its highly active surface adsorption. The fact that acetic acid solutions were necessary to remove the enzyme showed this adsorption; b. The enzyme was permanently denatured by the 80% acetic

acid. That this was probably incorrect was shown by previous experiments where acetic acid solutions of lysozyme reactivated upon lypholyzing to remove the acetic acid; c. When the enzyme in 80% acetic acid was mixed with the LZ pH 6.2 buffer-substrate suspension, the enzyme did not have time to renature from the form present in the 80% acetic acid. I feel that the last is the most probable explanation.

6. In this determination, the reaction conditions were essentially the same as in section (5) preceding. The lysozyme was desorbed with 33% acetic acid and the solvent was removed by lypholyzing. The enzyme was dissolved and allowed to renature in the LZ pH 6.2 buffer for several hours before assay. The material exposed in the reactor for one hour had less than 10% of its initial activity left while the control, treated the same except for exposure, had 60-70% of its original activity.

7. This determination was similar to the preceding two with work up as in (6), except that enough enzyme was recovered to perform amino acid analysis. The material oxidized for half an hour had an enzymatic activity of less than 10%, while the control had 64% activity. The amino acid analyses of these two samples, plus that of the starting material is shown in Table 6, under codes 709, 711 and 708, respectively.

Table 6. Lysozyme (Chicken eggwhite). Comparison of native enzyme and enzyme oxidized by microwave discharge generated singlet oxygen.

Amino Acid	Reported							
Analyzer	Standard	708-74	709-74	711-74	806-74	807-74	808-74	810-74
Run, Code								
residues								
His	1	0.9	0.18	0.9	0.6	--	0.9	--
Met	2	2.1	0.28	2.1	1.0	0.9	2.2	1.9
MetSO	0	0	0	trace	--	0.4	0	0.2
Cys	8	7.1	4.2	6.8	4.9	0.1	4.8	0.1
CySO ₃ H	0	0	0.5	trace	0.4	0.3	trace	0.4
Trp	6	5.3	1.4	5.8	<u>5.8</u>	--	6.1	--
Kyn	0	trace	1.9	0.0	<u>0.6</u>	--	0	--
Asp	21	21.2	22.7	21.1	24.3	13.7	23.6	14.4
Thr	7	6.9	7.5	6.9	7.2	0.1	7.3	0.2
Ser	10	9.5	9.9	9.7	10.5	1.7	10.4	2.3
Glu	5	5.0	5.0	4.8	5.2	2.2	5.2	2.0
Pro	2	--	2.2	--	--	--	2.0	0.7
Gly	12	11.9	12.2	12.2	13.3	16.9	13.5	15.6
Ala	12	11.7	11.8	11.9	13.2	15.4	13.3	16.2
Val	6	5.9	6.2	5.6	3.8	2.9	3.7	2.0
Ile	6	5.0	4.9	4.7	4.3	2.8	4.3	2.1
Leu	8	7.8	7.8	7.9	7.6	6.6	7.7	6.3
Phe	3	3.0	3.0	2.9	2.7	1.9	2.6	1.8
Lys	6	6.3	5.5	6.5	<u>4.5</u>	--	5.6	--
Arg	11	11.1	10.6	11.5	10.9	--	11.7	--
Ammonia	16	18.2	20.9	20.4	18.2	--	18.4	--
Tyr	3	3.0	2.8	3.0	3.0	2.3	3.0	2.5
Enzymatic								
Activity	100%	100	9	60	20	20	76	76

All hydrolyses used either 2 N methanesulfonic acid or 3.3 N sodium hydroxide. Basic residues were not determined in basic hydrolyses. The Ile value is the sum of Ile and alloIle in basic hydrolyses. Underlined values were determined by chart dot counting method when integrator failed. Lys and Tyr integrations overlapped and there is a tendency for the split to give high Lys and low Tyr. In 806, the Asp seems to represent both Asp and MetSO as they do not resolve well at high salt concentrations, as were used in this preparation.

The most obvious features of these results are the losses of His, Met, Trp and possibly Cys, with production of Kyn, cysteic acid (CySO_3H) and possibly methionine sulfoxide (MetSO). In the cases of the production of Kyn and CySO_3H , the amounts produced do not completely account for the loss of their parent compounds, Trp and Cys.

There is nothing surprising about these results. They do tend to confirm the observations of previous workers regarding the residues susceptible to singlet oxygen and indeed support the idea that singlet oxygen is the mediator of dye sensitized photooxidations of proteins.

8. This determination was made to confirm the results of the preceding section. Material oxidized for half an hour had a residual activity of 23% versus 76% for the control. The oxidation thus seemed somewhat gentler than in the preceding experiment, even though the time was the same and the support was stirred for better access to the oxygen stream. The chief differences that can be noted is that the loading of enzyme per g of support was between two and three times as high (ca. 7 mg/g vs. 3 mg/g) and the total amount of silica gel was three times as high in this determination. Perhaps the enzyme was not as well distributed as in the previous run or the presence of about three times as much silica gel in this run quenched the singlet oxygen stream too much. This last seems the most likely. This effect is discussed in more detail in the section on

violanthrone. The amino acid analyses of the oxidized and control material are shown in Table 6. Both methanesulfonic acid and basic hydrolyses are shown with codes of 806 and 807 for the acid and base runs with the oxidized material, respectively, and 808 and 809 for the control.

Again, no surprises are noted. The extent of oxidation shown seems to parallel that of the enzymatic activity. The main loss seems to be approximately one Met, with perhaps a third of the His and some Trp. Not much loss is noted in the Trp value shown, but the production of 0.6 Kyn shows some involvement.

9. When this study was extended to ribonuclease A, the results fairly well paralleled those obtained with lysozyme, even though some of the results were not as clear or satisfactory as with lysozyme.

Ribonuclease proved to be a less desirable experimental subject than lysozyme. The Gorin activity assay for lysozyme has some features which discourage its use in beginning biochemistry teaching laboratories, in spite of the fact that both enzyme and substrate are relatively cheap. The Kunitz ribonuclease assay is even more difficult, and, unfortunately, seems to be the best one available. Also, the enzyme and substrate are neither as inexpensive as with lysozyme so that a bucket chemist, like the author, cannot afford to throw it

around in preliminary experiments working out handling conditions. The only things that the enzyme had to recommend it for this study was that it is notoriously tough, as enzymes go, and is reasonably well characterized. However, there does seem to be some variation in commercially available material. Sigma lists several grades of varying purity and activity.

The main difference between this enzyme and lysozyme was the requirement for a somewhat stronger acetic acid solution to desorb and elute from the silica gel. A number of different conditions were tried. The best result, discovered and used with the material in section (10) following, was obtained with a stepwise elution using steps of 0, 33, 50, 66 and 80% acetic acid. If an adequate volume of eluent was used, 20 ml per step for three g of silica gel, nearly all of the elutable material will be found in the 33% fraction and the beginning of the 50% fraction where it is increasing to concentration. Done in this manner, very little material was observed in the late 50% or later fractions and overall yields seemed higher than with 80% alone. One brief study with lysozyme indicated that these conditions may be optimum there also. Even at best, though, no recovery from silica gel of more than about 80% was noted with either enzyme, usually less.

This first determination with ribonuclease-A used 80% acetic acid as an eluent in a single step. The oxidized material had no

detectable enzymatic activity. The control had an activity of about 60% relative to the starting preparation. This was comparable to the activity (55%) of material kept in 80% acetic acid and analyzed as the oxidized and control were. Since the Kunitz assay is run in 0.1 M pH 5.0, the elutant 80% acetic acid was not removed but only diluted and one half neutralized with sodium hydroxide to give a 1.2 M pH 5.0 acetate buffer. At the time of the assay, this is further diluted by 1:20 in 0.1 M buffer, but there may be residual effects of the low pH and high salt which had not had time to reconstitute in the assay. This may account for the low values observed with the controls. In the next section, the eluent was removed by lypholyzing, giving much higher activity values for the control.

Amino acid analyses were obtained on both the oxidized and control materials as shown in Table 7, codes 783 and 784, respectively. These were hydrolyzed by hydrochloric acid. A methanesulfonic acid hydrolyzate of the starting material is shown in 747, with part of a basic hydrolyzate shown in 748. Due to error on my part, only the five analyses shown in 748 were obtained.

Again, no surprises were noticed. His and Met appeared to be extensively damaged, the Met more than the His. There was difficulty with the integration of Cys in this sample so this datum is probably lost. This is the sole determination in all of this work which showed possible Tyr damage extensive enough to be probable.

Table 7. Ribonuclease-A (Bovine Pancreatic)

Lot Number		Sigma No. R-5125 lot 99B-8020				/	Sigma No. R-5125 lot 122C-8220			
Amino Analyzer Run Code	Reported Standard	747-74	748-74	783-74	784-74	802-74	803-74	804-74	805-74	
residues										
His	4	3.0	--	1.9	4.2	3.8	--	4.1	--	
Met	4	4.8	4.3	1.4	3.9	2.9	3.1	4.0	3.8	
MetSO	0	0	--	0.3	--	0	0.1	0	0.1	
Cys	8	4.4	--	=	5.4	5.8	0	6.9	0	
CySO ₃ H	0	0	--	0.5	0.4	0.2	0.7	0	0.1	
Tyr	6	5.6	5.6	4.1	5.1	4.9	6.3	5.5	4.9	
Asp	15	15.8	--	14.9	14.8	9.8	15.8	10.6	12.6	
Thr	10	10.1	--	9.7	9.5	10.8	0.7	10.2	0.4	
Ser	15	14.5	--	13.4	13.8	15.2	6.4	14.5	5.2	
Glu	12	12.4	--	12.4	12.1	3.4	6.5	4.2	7.1	
Pro	4	4.2	--	4.5	4.9	2.4	4.0	3.0	2.6	
Gly	3	3.8	--	3.7	3.6	3.1	16.1	3.0	11.3	
Ala	12	12.6	--	12.0	12.0	18.0	27.6	17.8	18.6	
Val	9	7.1	--	10.6	10.7	10.4	8.8	10.0	4.7	
Ile	3	1.6	1.8	2.3	2.5	3.0	2.7	2.2	1.5	
Leu	2	2.8	2.4	2.1	2.2	2.8	3.9	2.6	2.5	
Phe	3	2.9	3.1	3.1	2.9	2.9	3.6	3.0	2.4	
Lys	10	10.6	--	9.9	11.4	9.8	--	10.1	--	
Arg	4	5.0	--	3.5	3.7	8.2	--	7.3	--	
Ammonia	17	20.6	--	59.5	30.3	23.4	--	20.9	--	

747-74 - methanesulfonic acid hydrolyzate of beginning material

748-74 - basic hydrolyzate of beginning material

783-74 - hydrochloric acid hydrolyzate of material oxidized (microwave) for one hour, 0% of enzymatic activity

784-74 - hydrochloric acid hydrolyzate of control for 783, 60% enzymatic activity

802-74 - hydrochloric acid hydrolyzate of material oxidized for 0.5 hour, 31% enzymatic activity

803-74 - basic hydrolyzate of same material as 802

804-74 - acid (hydrochloric) hydrolyzate of control for 802, 116% activity

805-74 - basic hydrolyzate of same material as 804

10. This determination used ribonuclease under almost identical conditions of loading on the silica gel, its amount and time of exposure as the lysozyme work in section (8). The results were very similar also. In this determination, the eluent was removed by lypholyzing and the enzyme was allowed to renature in 0.1 M pH 5.0 buffer for several hours before analyzing for activity. The control had an activity of 116% relative to a sample of the original solution (that was added to the silica gel) which had been kept for two days in the refrigerator. The oxidized material had an activity of nearly 29% of this or about 31% relative to the starting solution. This particular assay run had some difficulties so that the exact percentages of relative activity may be somewhat in error although the relative 1:4 activity ratio for oxidized and control is well founded. By a fortunate happenstance, the concentration of the oxidized dilution for analysis was almost exactly four times that of the control, yet gave an activity slope exactly the same. The main uncertainty is just how active that was.

The amino acid analyses, acid and base for the oxidized and control, respectively 802, 803, 804 and 805 in Table 7, showed relatively little damage. The only particularly notable deletion is the loss of one Met, with a poor correlation for product sulfoxide. Again, a slight diminuation of the Tyr is possible, but not too probable. The slight effect on the His is somewhat surprising.

Table 8. Amino acid analysis of Ribonuclease-A

Amino Acid Analyzer Run Code 822-74					
Residues	Reported Standard	822-74	Residues	Reported Standard	822-74
Lys	10	11.6	Ala	12	12.5
His	4	4.1	Cys	8	6.9
Arg	4	3.6	Val	9	8.8
Asp	15	15.5	Met	4	4.2
Thr	10	10.1	Ile	3	2.4
Ser	15	13.6	Leu	2	2.0
Glu	12	12.8	Phe	3	3.0
Pro	4	4.1	Tyr	6	6.0
Gly	3	3.5	Ammonia	17	30.9

Hydrochloric acid hydrolyzate of native anzyme from lot 122C-8220

The main difficulties with this set of analyses are the 50% excess values shown for Ala and 100% excess for Arg with parallel losses of Glu and perhaps Pro. Table 8 lists the results of the analysis of enzyme from the same lot as the questionable results using the same reagents. As the results are in accord with the known analysis of ribonuclease-A, the wild values for that block of analyses remains inexplicable.

The main conclusions that can be drawn from this section are as follows:

- a. An oxidation in dry bulk of proteins by singlet oxygen is difficult to perform.
- b. Simple dispersion on a support which does not strongly interact with the enzyme does not increase the facility of the reaction very much.
- c. Silica gel may have a catalytic role in addition to supporting and dispersing the enzyme. Further work would be necessary to clarify this point.
- d. Protein containing formations, i.e., people, probably have little to fear from atmospheric singlet oxygen.

C. Violanthrone. Small amounts of this blue dye deposited on a dispersant yields a vivid red chemiluminescence in the microwave reactor. All of the supports listed in the Methods section regarding

violanthrone would serve except for the MN-300 microcrystalline cellulose thin layer chromatographic sheets. The reason for this exception is not certain. Free microcrystalline cellulose powder works well as does filter paper and several types of chromatographic cellulose. The binder used in the MN-300 is now known, but it would seem that it is a powerful singlet oxygen quencher.

When the dye was deposited in a thin film on glass, no reaction could be noted. Whether this is due to low surface area or some type of intermolecular quenching phenomena is unknown.

As an indicator of singlet oxygen presence, violanthrone proved to be very useful. Using a tuning spot of the dye on a small piece of filter paper inside of the lid of the reactor flask permitted the following observations about the operation of the reactor to be made:

1. Optimum production of singlet oxygen was observed at 1-3 torr of pressure in pure oxygen and at 10-20 torr in 10% oxygen in helium.
2. It made little difference in the power setting of the generator as long as a discharge could be maintained. Settings of lower than 20% power gave an unstable discharge so operation was usually in the 20-40% power range.
3. Silica gel, either in sheets or in bulk, severely quenched the singlet oxygen. This presents an apparent paradox.

While oxidation of substrates was efficient only on silica gel, it rapidly inactivated the oxygen. The report of Scheffer and Ouchi (1970) mentioned that both silica gel and microcrystalline cellulose were effective supports and could determine little difference between them. They expressed some surprise at this as they noted that silica gel is a much stronger quencher of singlet oxygen than the cellulose. I was able to observe little difference between the two absorbants with the violanthrone reaction as long as only a gram or so of the powder was present. When the amount of silica gel was increased or if a full 20 cm wide sheet of silica gel thin layer chromatographic sheet was present, then notably less luminescence could be observed. No such effect seemed to be noticable with bulk cellulose powders. An aspect of this work that is still puzzling is that violanthrone is reactive, although apparently not oxidized, on both cellulose, including paper, and silica gel while oxidations of methionine and lysozyme are possible only on the silica. A major difference is that the violanthrone is present in amounts that can be estimated to be on the order of nanograms per square cm while the others are present in larger amounts, e.g., about $500 \mu\text{g}/\text{cm}^2$ for a typical methionine spot. This aspect of dispersion may be all that is involved, although at this time, not enough information is available to say.

Demonstration of Photooxidation with the Clark Electrode

Since the data obtained here with the Delta Scientific field instrument are generally unsuitable for precise quantitative purposes, I will note only whether a particular reaction is fast or slow, relative to the photooxidation of His sensitized by Rose Bengal and list a typical "oxygen consumption coefficient" (OCC) of decrease in relative scale units per minute. I don't feel more detailed presentation of this data is warranted.

A. Amino acids

1. Rose Bengal with His. This oxygen consumption reaction was shown to be dependent on a light and dye. The OCC for the conditions described was about 10.

2. Cystine and Rose Bengal. This reaction was shown to be light dependent. As CysH is known to be slowly oxidized in solution to the disulfide, this point was especially examined. Dark oxygen consumption was negligible. This reaction is somewhat slower than the His case. The OCC was about 8.

3. His with Crystal Violet. No reaction, light dependent or otherwise could be demonstrate- with this system. The OCC was less than 0.1, which is about the limit of sensitivity of the instrument.

4. CysH with Crystal Violet. A very slow light dependent reaction could be observed. The OCC was about 0.3.

5. CysH with Crystal Violet. This used less CysH and more crystal violet than the preceding section. Again a slow reaction was noticed. The OCC was about 0.3-0.5. There seemed to be a residual light dependent effect. Before the light was turned on, no consumption could be observed in an hour. After about half of the oxygen in the solution was consumed, the light was turned off and the consumption continued at a slower rate, 0.2 decreasing to 0.1 in an hour and a half. It went back up to 0.3 when the light was turned back on.

6. CysH with Crystal Violet. This used the same reagent concentrations that were used in (4). The difference was a much brighter light. The reaction is still slow, but it did speed up to an OCC of about 0.7-0.8. The same residual effect was noticed here as in (5). The rate of this varied from 0.4 to 0.2.

7. His with Proflavin. This reaction was strictly light dependent. No residual effect was noticed as with CysH and crystal violet. Even though it used a brighter light, it was not quite as fast as His with Rose Bengal. The OCC was about 7.0.

8. Cys₂ with Rose Bengal. It took a while for the instrument to settle down in the dark with this. After it stabilized, a

slow light dependent reaction of OCC about 0.5 was observed. No dark residual could be observed.

9. Cys₂ with Rose Bengal. As in the preceding section, with better instrument control. The OCC was about 1.0.

10. Cys₂ with Rose Bengal. The same solution as in (9) was reoxygenated and rerun. The OCC was about 0.8. The solution was exposed for about half an hour after instrument stability was reached. The pink of the dye faded and yellowed and would not reconstitute when reoxygenated.

11. Cys₂ with Rose Bengal. More dye was added to the yellowed solution and reilluminated. Consumption was completely light dependent. When the light was on, the OCC was 1-2. It was zero when the light was off.

12. Alanine with Rose Bengal. No consumption could be observed in an hour.

13. Met with Rose Bengal. While this is not directly comparable with the previous experiments in this series since the temperature and volume are significantly different, the reaction is fast with an OCC of about 11.

B. Photooxidation of pyrocatechol. The reaction of pyrocatechol sensitized with rose bengal seems fairly slow. The OCC was about 1-2 in the first run described and about 1.5-3.0 in the second. The reaction is dependent on both light and dye.

The conclusions that can be drawn from all of this are the following:

1. Of the amino acids tested, His, Met, CysH, Cys₂ and Ala, the reactivities observed for sensitization by rose bengal and proflavin are about what would be expected from various reports in the literature (Weil, 1951, 1965; Sluyterman, 1962).

2. The specificity of sensitization by crystal violet for cysteine supports the reports of Jori et al. (1969) and Bellin and Yankus (1968). The reaction seems unlikely to be a Type II singlet oxygen reaction.

3. Pyrocatechol can be photooxidized if sensitized by rose bengal. As far as I know, the dye sensitized photooxidation of pyrocatechol has not been previously reported, although other phenol type compounds are known to be susceptible (Saito and Matsuura, 1970).

When this work was started, the intention was to extend this type of study to the deuterium oxide/azide type of test on some of these substrates and investigate more fully the specific reaction of crystal violet.

Spectral Studies of the Interaction of Hematoporphyrin and
Lysozyme

My attempts to verify the observations of Jori et al. (1971) of interactions between lysozyme and hematoporphyrin, which change the absorbtion spectrum of the dye, were unsuccessful.

At pH of 6.2, comparable to the 5.9 value Jori used, a 10 μM solution of hematoporphyrin showed a maximum centered at 375 nm. When lysozyme was added, under 0.1 M buffer conditions permitting little change in pH, no shift in the absorbance maximum wavelengths was observed. No difference was observed at different concentrations, e.g., 5 μM . No difference was observed in this result by changing lots of lysozyme, sources of hematoporphyrin or preparation of the reagents. Jori et al. reported that the mixing of 5 μM lysozyme with 5 μM hematoporphyrin at pH 5.9 shifted a rather sharp maximum from about 388 to 405 nm.

I did observe that, while the presence of equimolar or even 10 times excess lysozyme had little if any effect on the hematoporphyrin spectrum, changes of pH did. There seems to be several spectrally distinct species, depending on the degree of protonation. Table 9 lists the maximum wavelengths and solution conditions observed.

Table 9. Hematoporphyrin $\lambda_{\max}$ at Various pHs

Solution	Maximum	Width of Maximum
0.05 M NaOH	392	medium
pH 8.5 water	380	broad
pH 7.2 water	378	broad
pH 6.5 phosphate buffer	375	broad
pH 5.2 phosphate buffer	374	medium
pH 4.8 phosphate buffer	375	medium
87% acetic acid	403	very narrow

Lysozyme, in varying concentrations, was added to several of these, as detailed in the Methods section. At no time was a spectral shift observed, unless the pH changed.

The only conclusion that I can draw from this is that either Jori or I did something wrong. Solutions 10 μM in lysozyme are very prone to foaming, yet they mention flushing the solutions in two cm cuvettes with oxygen. They mention no antifoaming agent, but I have wondered if they might have used one, causing the results they report.

As mentioned earlier, in the section on the hematoporphyrin sensitized photooxidized lysozyme, some type of interaction does take place between the dye and the protein, particularly at higher concentrations. Whether this is true or not at 5 and 10 μM is not certain as solutions containing that concentration of both dye and protein

are stable and do not precipitate at pH 6.2. There are unresolved questions here which, hopefully, someday can be settled.

Oxidation of Tryptophan by Singlet Oxygen Generated by Non-Dye Means

A. Rose Bengal sensitized photooxidation of N-Glycyltryptophan. This photooxidation was done to provide a basis of comparison for oxidations performed by other means. The main point of the experiment was the ultraviolet absorption spectra, as that was the means used for monitoring Trp reaction in the rest of this section. It was deemed useful to additionally make chromatographic comparisons of the product with kynurenine to establish the nature of the product in this series and in others where Trp and peptides containing it are oxidized.

The following table lists the spectral results observed. The absorbances listed at specific wavelengths were determined using the DU-2. Spectral peaks were observed with the Cary-14, seemed to be able to resolve 3-5 nm at 280 nm.

Table 10. Absorbances and peak absorbance wavelengths

	Peaks	A ₂₄₀	A ₂₆₀	A ₂₈₀	A ₃₆₀	A ₂₈₀ /A ₂₆₀
Typ (1.5 × 10 ⁻⁴ M)	280, 288	.294	.638	.940	.004	1.47
Kyn (10 ⁻⁴ M)	256, 360	.768	.616	.043	.385	.07
NGT (10 ⁻⁴ M)	280, 288	.158	.264	.435	.003	1.65
DSPO NGT (10 ⁻⁴ M)	260, 320	.540	.400	.087	.019	.224
Hydrolyzate of DSPO NGT	258, 360	.705	.508	.087	.300	.171

Table 11. Thin layer chromatographic results

	R _f	BAW	R _f	MAC	(× 100)	Color with	
						Ehrlich's	Ninhydrin
Typ	52;	53	57;	55		blu	pur
Kyn	48;	49	56;	57		yel	pur
NGT	47;	46	54;	55		blu	yel
DSPO NGT	46;	37;	61,	56,	--;	yel, yel	yel, yel;
	42,	36	62	57,	(46)		(blu)
Hydrolyzate of DSPO NGT	46,	--;	56,	--;		yel	pur, pur
	49,	25	57,	41			
Gly	--;	23	--;	35		--	pur
Met	--,	46	--;	59		--	pur

blu = blue, yel = yellow, pur = purple

BAW is Butanol-Acetic acid-Water(4:1:1) by vol., MAC is Methanol-14% ammonium hydroxide-Chloroform(2.1:1:2) by vol., both run on EK 13191 prescribed silica gel with fluorescent indicator. Kyn and the BAW 46-49 spots and MAC 56-57 spots from both DSPO NGT and its hydrolyzate were blue fluorescent. The two R_f values given in each entry are respectively the value observed with the Ehrlich's development and the Ninhydrin development.

The amino acid analysis of the hydrolyzed photooxidized NGT yielded peaks at 13, 20 and 41 minutes (short column). Kyn standard appears at 20 minutes and ammonium at 39 minutes. The material at 13 minutes was assumed to be Gly. The integrations calculated as 1.00 residue of Gly, 1.04 residue of Kyn and 0.47 residues of ammonium.

The conclusions that were drawn from this are that Gly Trp is photooxidized to a GlyKyn derivative, presumably N'-formyl-Kyn, which has absorption maxima at 260 and 320 nm as opposed to the 280 nm of Trp and 258 and 360 nm of Kyn. The N'-formyl-Kyn peptide is not very fluorescent while Kyn itself is. The GlyKyn derivative can be hydrolyzed to equimolar amounts of Gly and Kyn. All of this supports the reports in the literature about the photooxidation of N-blocked-Trp (Benassi et al., 1967; Savage, 1971). This also showed that photooxidation of Trp is accompanied by shifts in the 280/260 absorbance ratios. This can then serve as a quick and easy monitor for oxidation of Trp.

B. Oxidation of tryptophan by hydrogen-peroxide-hypochlorite generated singlet oxygen. When Trp was exposed to singlet oxygen generated by the reaction of hydrogen peroxide and sodium hypochlorite, a reaction could be observed. The 280/260 ratios for starting material, a control using only hydrogen peroxide and the complete

mixture were 1.40, 1.43 and 1.11, respectively. This indicates some reactivity, but as the condition of the reaction should have yielded a three molar excess of singlet oxygen over Trp and the ratio observed with an essentially complete reaction is about 0.2-0.4, the Trp would not seem to be a very good substrate for a singlet oxygen reaction.

While this was a notably positive result, no further work was done with this system since proteins have been reported to be reactive to hydrogen peroxide alone, particularly at Met. I was recently informed by Dr. Phillip Madden (1974) that his laboratory at the University of Puget Sound had inactivated an enzyme by this reaction, showing significantly higher reaction with the complete mixture than with either peroxide or hypochlorite alone.

C. Oxidation of tryptophan and lysozyme by potassium perchromate generated singlet oxygen

1. Trp was oxidized, as described in the Methods section, presumably by singlet oxygen generated by the decomposition of potassium perchromate (more properly, peroxychromate). This method of generation had some problems as the decomposition produced yellow chromate ion as a by-product. Unfortunately, this absorbs slightly at the wavelengths of interest. To overcome this problem, a reagent blank was prepared from potassium chromate and the contributions at

the analytical wavelengths were subtracted from the values observed in the oxidation solutions. In a control experiment, Trp was exposed to slightly higher chromate concentrations than the solution with the peroxychromate had upon decomposition. The following data were obtained:

Table 12. Absorbance ratios of tryptophan oxidized by peroxychromate generated singlet oxygen

	A_{260}	A_{280}	A_{280}/A_{260}
Trp (6×10^{-3} M)	.288	.422	1.46
Control (6×10^{-3} M Trp, 6.5×10^{-3} M $\text{CrO}_4^{=}$)	.436	.580	--
Blank (6.5×10^{-3} M $\text{CrO}_4^{=}$)	.216	.237	--
Oxidized (6×10^{-3} M Trp, 4.25×10^{-3} M $\text{CrO}_4^{=}$)	.493	.478	--
(Control) - (Blank)	.224	.343	1.50
(Oxidized) - (0.65 Blank)	.255	.224	0.88

It was concluded that specific oxidation was observed. It is not entirely clear what the exact mode of decomposition of the peroxychromate is. From the structural formula, $\text{K}_3\text{CrO}_2(\text{O}_2)_3$, containing three moles of peroxide and pentavalent chromium, it would seem possible that two moles of oxygen can be released. When the conditions were chosen for this experiment, I assumed that this would generate

two moles of singlet oxygen, so the peroxychromate: Trp ratio of 8.4:12 would provide a good excess of oxygen over Trp for reaction. However, a later conversation with Dr. Peters (1972) indicated that only one mole of singlet oxygen was released, and then only under reasonably alkaline conditions. One point should be kept in mind in this discussion: While the reagent is known to produce singlet oxygen and chromate does not particularly affect Trp, nothing presented here rules out a direct (heterogeneous) reaction between the reagent and Trp having nothing to do with singlet oxygen.

2. When this oxidation was extended to lysozyme, little useful information was learned. A reaction took place, however, it denatured the protein so extensively that it formed a yellow precipitate which could not be redissolved or decolorized. A control with lysozyme and chromate did not precipitate.

CONCLUSIONS

The products of oxidation by either microwave generated singlet oxygen or a dye sensitized photooxidation of several amino acids were shown to be identical. Amino acids known to be susceptible to dye sensitized photooxidation include methionine, histidine, tryptophan, tyrosine and cysteine-cystine. Of these, methionine and derivatives of histidine--N-benzoylhistidine--and tryptophan--N-glycyl and N-acetyltryptophan--were oxidized in solution by microwave generated singlet oxygen and by dye sensitized photooxidation using rose bengal or methylene blue as sensitizers.

For each substrate, the products of dye sensitized photooxidation using both methylene blue and rose bengal were found to match the products of the microwave reaction. This exact correspondence between products was determined using a variety of solvents and chromatographic materials.

The apparent identity of the products of dye sensitized photooxidation of these amino acids provides direct evidence for the mediation of singlet oxygen in the dye sensitized photooxidation of amino acids and proteins.

Coupled with the work of Nilsson et al. using either a singlet oxygen quencher or enhancer which showed that the quantum efficiency of photooxidation is dependent on the steady state concentration of singlet oxygen, this result demonstrates the conclusion that singlet

oxygen mediates typical dye sensitized photooxidations of proteins and amino acids.

The results of oxidation, in the dry state, of amino acids, primarily methionine, and the proteins lysozyme and ribonuclease by singlet oxygen showed that a very high surface area is necessary for much reaction to take place. The amino acids, both free and in the proteins, which were oxidized by the microwave generated singlet oxygen paralleled those of dye sensitized photooxidation. Where products could be identified, with the possible exception of N-benzoylhistidine, they corresponded to those of photooxidation in aqueous solution.

The effects of dispersion on substrates for dry microwave generated singlet oxygen oxidations have aspects which suggest that not only a very high surface area is required but possibly a surface interaction with silica gel is also necessary for substantial reactions to occur. The inertness to reaction by proteins and amino acids in bulk with discharge generated singlet oxygen suggests that free atmospheric singlet oxygen may represent a very limited hazard to proteins.

The study of the hematoporphyrin sensitized photooxidation of lysozyme shows that the majority of the reaction is mediated by singlet oxygen. This system was examined to see if it provided a highly specific protein modification ability and an example of a non singlet oxygen Type I dye sensitized photooxidation. It was a disappointment when it showed neither.

Using the methods of Nilsson et al. with photooxidations in the presence of either deuterium oxide or azide, inactivations were much more vigorous in the presence of D_2O and much less in the presence of azide. The residues which were attacked in the presence of azide or D_2O were not apparently different from those destroyed in water, except for the extent of oxidation. These results are not consistent with reports of Jori et al. where highly specific and limited amino acid oxidations were noted. The specialized dye binding and photooxidations described by them for the hematoporphyrin-lysozyme system could not be duplicated in my hands.

In conclusion, an additional bit of understanding of a fundamental effect of nature, dye sensitized photooxidation, has been achieved.

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