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QUANTUM YIELD STUDIES OF THE
PHOTODYNAMIC INACTIVATION OF TRYPSIN

by

Blaine W. Glad and John D. Spikes

Technical Report

U.S. Atomic Energy Commission Contract No. AT(11-1)-875

Department of Molecular Biology

University of Utah
Salt Lake City 12, Utah

June 1964

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Meaning</u>
BAEE	Benzoyl-L-arginine ethyl ester
MeB	Methylene blue
FMN	Flavin mononucleotide
AET	S-(2-aminoethyl)isothiuronium bromide hydrobromide
E_a	experimental energy of activation
kcal	kilocalories
CMU	3-(p-chlorophenyl)-1,1-dimethylurea
u	micro
<u>M</u>	molar
gm	gram
ml	milliliter
cm	centimeter
min	minute
sec	second
OD	optical density
t	time
l	liter
ul	microliter
$^{\circ}\text{K}$	degrees Kelvin
$^{\circ}\text{C}$	degrees Centigrade
λ	wavelength
v_o	initial velocity
Φ	quantum yield
$\text{\AA}$	angstroms
mu	millimicrons

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I. INTRODUCTION

A. Photodynamic Action

Photodynamic action is operationally defined as the chemical alteration of a molecule or the damaging or killing of a cell or multicellular organism by light in the presence of an appropriate sensitizing dye and molecular oxygen. Oxygen is consumed in the process. "Light" is taken to mean that portion of the electromagnetic spectrum between about 300 mμ and 800 mμ. Wavelengths much shorter than 300 mμ (in the ultraviolet region) cause chemical changes directly in molecules of biological importance such as proteins and nucleic acids, and those much longer than 800 mμ (in the infra-red) have never been shown to be effective in photodynamic action. The sensitizing "dye" is defined for the purpose of this paper as any compound which can trap energy in this region of the spectrum in such a way that it can be used for photodynamic action. Effective compounds include many, but not all, of the standard types of dyes of the organic chemist, a number of carcinogenic hydrocarbons, flavins such as riboflavin and flavin mononucleotide, chlorophyll and certain of its derivatives, certain porphyrins, and a variety of other types of compounds (1).

Photodynamic action was discovered by Raab (2) in 1900. He found that the dye acridine orange sensitized paramecia to killing by light. Light alone had no effect and dye in the dark had no effect. Marcacci (3) published some observations along these lines even earlier. Tappeiner (4) enlarged on the work of Raab and coined the term "photodynamic action."

Since this early work on photodynamic action around the turn of the century, many hundreds of studies have been made on the phenomenon. Experimental materials have included many types of simple and complex molecules (5), single-celled organisms (6), and multicellular organisms (both plant and animal), all the way up to man himself (7). Molecules as simple as phenol (8) and certain amino acids (9-11) can be altered by photodynamic treatment. More complicated molecules reported to be altered photodynamically include proteins (12-15) and nucleic acids (16). Viruses can be modified or inactivated photodynamically (17-19). Isolated subcellular particulates such as chloroplasts can be photodynamically inactivated (20), as can the photosynthetic apparatus of intact cells (6).

The aromatic amino acids tyrosine, tryptophan and dihydroxyphenylalanine, as well as the non-aromatic amino acids methionine and histidine, can be rapidly altered by photodynamic treatment. In all such cases, photooxidation occurs, with the ring structures being opened in the aromatic amino acids (9-11). The aliphatic amino acids are resistant to photodynamic action.

Proteins containing the amino acids listed above can be photodynamically altered. Enzymes have been used for many studies of this type since effects on catalytic activity can be measured. Photodynamic effects on many different enzymes, including α -amylase, carbonic anhydrase, chymotrypsin, cytochrome-c, dehydrogenases, hyaluronidase, lysozyme, myosin, pepsin, peroxidase, phosphoglucosmutase, carboxypeptidase A, ribonuclease, urease, catalase, and enolase (12-15), have been studied. All of the original work reported here has been carried out with trypsin. This enzyme was selected since a considerable literature exists on its inactivation

by ultraviolet and gamma radiation. Further, it is readily obtainable, easy to handle, and several simple assays for its activity are available. Three sensitizing dyes were chosen for this study: methylene blue, eosin Y, and flavin mononucleotide.

The subject of photodynamic action was reviewed by Blum (21) about two decades ago. Since that time, several modern reviews have been written that cover the subject thoroughly (14-15,22-26). Certain aspects of the field, especially effects on proteins, enzymes and amino acids, were reviewed extensively in an earlier thesis from this laboratory (12).

Although this thesis is concerned only with photodynamic action, it should be stressed that some dye-sensitized processes do not require molecular oxygen. For example, 8-methoxypsoralen can sensitize the photodegradation of enzymes under anaerobic conditions (27). Thus, photodynamic action is concerned with only one small segment of dye-sensitized photochemistry.

A large number of kinetic mechanisms have been proposed to account for photodynamic action (26,28-36). Those most commonly accepted at present may be summarized as follows for the case where protein or enzyme is the substrate:

where D is the sensitizing dye, $h\nu$ is a photon of light, * indicates a short-lived excited state, ' indicates a long-lived excited state, O_2 is molecular oxygen, P is a protein molecule, PO_2 is an oxidized form of the

protein, DO_2 is a photoperoxide of the dye, and DH_2 is a reduced form of the dye.

Mechanism (I) postulates that the dye is raised to a short-lived excited state (probably the first excited singlet state), which undergoes intersystem crossing to a metastable or long-lived excited state (probably the triplet state). This interacts with molecular oxygen to form a photoperoxide, which reacts with the protein to produce an oxidized protein plus free dye in the ground state. This, at present, is the most commonly accepted mechanism (28). Mechanism (II) involves the same initial steps but postulates that the dye in the triplet state interacts with the protein to produce an excited protein molecule which then interacts with molecular oxygen to give the oxidized protein. Mechanisms (I) and (II) cannot be distinguished kinetically (28).

Mechanism (III) postulates an initial binding between dye and substrate followed by the photoactivation of this complex to the triplet state. This then reacts with molecular oxygen to give the oxidized protein plus the free dye in the ground state. This mechanism has some support because dye-protein complexes have been demonstrated under certain conditions (37).

Mechanism (IV) postulates the usual pathway of activation of the dye into the triplet state, followed by the concomitant reduction of the dye and the oxidation of the protein, after which the dye is reoxidized to the free ground state by molecular oxygen with the formation of hydrogen peroxide. Hydrogen peroxide is produced in some photodynamic systems but only in very small amounts (38). In earlier studies on the dye-sensitized photoinactivation of trypsin, it has been shown that none of these mechanisms fits the data precisely.

A very simplified schematic diagram intended to show some of the overall relationships felt to be involved in photodynamic action is shown in Fig. 1. In this scheme, the dye molecule is first excited to the singlet state; it may then cross over to the long-lived excited state (triplet), or it may return to the ground state with fluorescent emission. Once in the triplet state, it may also return to the ground state by emitting phosphorescent light, or it may participate in the reactions involved in oxidizing the substrate, consuming molecular oxygen and regenerating the dye in the ground state. The diagram in Fig. 1 does not suggest any mechanism for these processes.

Some dye destruction or alteration (photobleaching) is seen in certain photochemical systems, but this is apparently not involved to any great extent in photodynamic systems. In some cases, the coupling to the substrate alteration reaction may take place through the singlet rather than the triplet state (39).

B. Quantum Yield Measurements

Definition of quantum yield - In general, the quantum yield, Φ , of a photochemical reaction is defined as:

$$\Phi = \frac{\text{number of chemical alterations produced}}{\text{number of quanta of light absorbed}}$$

In the present photodynamic system, under any given conditions, the number of trypsin molecules inactivated divided by the number of quanta of light absorbed by the sensitizing dye is defined as the quantum yield:

$$\Phi = \frac{\text{number of trypsin molecules inactivated}}{\text{number of quanta of light absorbed}}$$

Fig. 1. A simplified schematic diagram of photodynamic action. Light activates the dye to the short-lived excited state (singlet), followed by its intersystem crossing to the long-lived excited state (triplet). The energy of the dye in the triplet state is then used in some way to produce an oxidized substrate with the dye returning to the ground state. Molecular oxygen is used up in the process. No inferences are intended as to the order in which the dye, molecular oxygen, and substrate interact. The fluorescent and phosphorescent pathways represent non-productive transitions of the dye back to the ground state. In some cases, the coupling of the dye to the substrate alteration reaction may take place through the singlet rather than the triplet state, as explained in the text; dye binding to the substrate may or may not occur.

The quantum yield is a dimensionless number and hence one can compare the photochemical efficiencies of different systems. In the present investigation, we desired to compare the quantum yields for the photodynamic inactivation of trypsin with several different sensitizing dyes as measured under a variety of reaction conditions in order to learn more about the reaction mechanisms. In some areas of photobiology, such as photosynthesis, the quantum requirement, the reciprocal of the quantum yield, is reported.

The above definition for quantum yield is not directly useful in the present system because the enzyme inactivation reaction is first-order (exponential) and hence the number of trypsin molecules inactivated per unit time changes with time of illumination. To overcome this problem, the quantum yield also can be obtained by dividing the rate of trypsin breakdown by the rate of absorption of quanta of light:

$$\Phi = \frac{dS/dt}{dQ/dt}$$

where S stands for the substrate (in this case trypsin), Q stands for quanta of light, and t is for time. Since dS/dt changes with time, we measure the initial breakdown rate and divide this by the initial quanta absorption rate:

$$\Phi = \frac{(dS/dt)_0}{(dQ/dt)_0}$$

where the subscript 0 stands for zero time after illumination; $(dS/dt)_0$ can be shown to be equal in the trypsin system used here to E_0/t_{37} ; E is the initial trypsin concentration; and t_{37} is the time at which thirty-seven per cent of the original enzymatic activity remains (see section A of Results and section F of Materials and Methods).

The light energy absorbed can be measured directly and converted into dQ/dt since the light is essentially monochromatic and the following relation holds:

$$E = hc/\lambda$$

where E is the energy per photon, h is Planck's constant, c is the velocity of light, and λ is the wavelength of the light (see sections E and F of Materials and Methods).

Quantum yield ranges - Quantum yield measurements have been made on a large number of photochemical systems, since such data not only define the photochemical efficiency of a reaction, but also may indicate much about the actual reaction mechanisms involved. In the simplest case, a quantum yield of one would indicate that one molecule is chemically altered for every quantum of light absorbed. This does occur in a few systems. Quantum yields greater than one indicate that one quantum of absorbed light can alter more than one substrate molecule; this occurs in those cases where the absorption of a quantum triggers a chain reaction. Once the process has been started, it is self-perpetuating. The quantum yield then shows an efficiency in this case out of proportion to the actual triggering reaction which occurred. Quantum yields of 100 - 1,000 are not uncommon.

Quantum yields lower than one are observed most commonly and indicate that part of the absorbed energy is not being used for the chemical alteration being observed. In addition to driving particular chemical reactions, electronic excitation energy may be given off as fluorescence, delayed fluorescence, or phosphorescence, or may be degraded to heat. Further, in studies with enzymes, some of the chemical reactions could be

non-productive in that the energy might bring about changes in the molecule which would have no effect on its catalytic activity.

The quantum yield of a photochemical reaction is often changed markedly by reaction variables such as dye concentration, light intensity, pH, etc. It is hoped that a critical, quantitative examination of the quantum yield of the photodynamic inactivation of trypsin as studied with several dyes as a function of such variables will permit a more definitive evaluation of the proposed reaction mechanisms for the process.

Quantum yields for fluorescence and phosphorescence - In many photochemical systems, relatively large fractions of the absorbed energy are lost as fluorescence and phosphorescence. This severely decreases the amount of energy available to drive chemical reactions. The quantum yield for fluorescence or phosphorescence is defined as follows:

$$\Phi = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}$$

In both simple organic systems and with dyes, the quantum yields for fluorescence and phosphorescence can be large. Quantum yields of this type have been measured in a large number of systems. However, only a few examples can be given here to illustrate the magnitudes of the energy lost in this way. For example, Melhuish (40) reports the following values for the quantum yields of fluorescence of various organic compounds as indicated: 1×10^{-3} M diphenylhexatriene in H_2SO_4 (0.750); 5×10^{-3} M anthracene in benzene (0.508); 2×10^{-3} M perylene in benzene (0.800); and 8×10^{-3} M anthranilic acid in benzene (0.536). In some of these examples, most of the absorbed energy is lost as fluorescence. Of more direct importance to the present work, quantum yield measurements of the fluorescence

of many photodynamically active dyes have been reported. Some examples by Weber and Teale (41) include the following: fluorescein in water (0.65); eosin in 0.1 M sodium hydroxide (0.19); acriflavin in water (0.56); riboflavin in water (0.26); chlorophyll a in benzene (0.33); and chlorophyll b in benzene (0.11). Here again, fluorescence can be relatively efficient. In the case of fluorescein, for example, over sixty per cent of the absorbed energy is emitted as fluorescence and is therefore unavailable for photodynamic reactions.

Quantum yields of sensitized photochemical reactions - Most of the systems studied in ordinary photochemistry involve reactions in which the molecule under primary consideration absorbs the light directly. For example, acetone can be oxidized photochemically by illumination with ultraviolet radiation of wavelengths absorbed directly by the acetone (42). In the present case, however, we are concerned with photochemical reactions of the type in which the photons are absorbed by a sensitizer and then energy is transferred in some way to the substrate molecule under consideration. Sensitized photochemical processes can occur in rather simple systems. For example, the mercury-sensitized photodecomposition of simple hydrocarbons by 2537 Å⁰ ultraviolet radiation has been studied (43). Discher et al. (44), have measured the quantum efficiency of the uranyl oxalate actinometer in which the uranyl ion absorbs the light and sensitizes the decomposition of the oxalate. At 365.5 mμ the quantum yield is 0.490 - 0.497, while at 435.8 mμ the quantum yield is 0.566 - 0.578, depending on the usual kinetic variables.

Some studies have been made on the quantum yields of anaerobic dye-sensitized reactions. For example, Bellin and Oster (37) measured the

anaerobic photoreduction of eosin. Eosin, bound to polyvinylpyrrolidone (PVP), has a higher quantum yield (approximately 0.006) of photobleaching in the presence of ascorbic acid than when free. In our trypsin system, it is not known whether the dye must be bound to the protein for photodynamic action to occur (12-13). Pestemer (45) has measured the quantum yield of the anaerobic photochemical reduction of thionine at 25° C in buffered fifty per cent ethanol. The electron donors used together with the corresponding quantum yields were anethole (0.09 - 0.26), diethylallylthiourea (0.08 - 0.21), and allylthiourea (0.09 - 0.17). In all cases, the quantum yields increased with decreasing pH and electron donor concentration.

Quantum yields of photodynamic action - Relatively few measurements of quantum yields in photodynamic systems have been made. This reflects the fact that this area is only a small subdivision of photochemistry; further, such measurements are tedious and difficult to make accurately. Spealman and Blum (46) have measured the quantum yield of the photodynamic conversion of iodide to iodine as a function of light intensity, oxygen partial pressure, pH, substrate concentration, etc., using rose bengal and eosin as the sensitizers. Values ranging around 0.06 were found, which are considerably larger than those for the photodynamic inactivation of enzymes. The non-sensitized photochemical conversion of iodide ion to iodine by ultraviolet light, in contrast, has a larger quantum yield of 0.15 (47).

Gaffron (48,49) found quantum yields of nearly unity for oxygen uptake in systems involving the chlorophyll- and mesoporphyrin-sensitized oxidation of molecules such as allylthiourea. With rubrene, which forms a photoperoxide on illumination in pyridine and benzene, the quantum yields

based on oxygen uptake are also near unity (49). This has also been shown by Bowen and Steadman (50). No comparable quantum yields for oxygen uptake during the photodynamic inactivation of enzymes have been published.

Uchida (51) has measured the quantum yield for the acridine-sensitized photooxidation of leuco-uranine (sodium fluorescein). The yield for the formation of uranine from leuco-uranine is 0.0070, while that for the decomposition of leuco-uranine is 0.0055. These two reactions are competitive under certain conditions.

Shugar (52) determined quantum yields for the riboflavin-sensitized photodynamic inactivation of indoleacetic acid and tryptophan; the values were 0.234 for indoleacetic acid and 0.038 for tryptophan. Shugar (52) also studied photodynamic effects on lysozyme and ribonuclease; the former was inactivated photodynamically by riboflavin, while the latter was not. He interpreted these results as implicating tryptophan destruction in the riboflavin-sensitized photoinactivation of enzymes, since this amino acid is present in lysozyme and absent in ribonuclease. In contrast, Ghiron (12) and others (53) have more recently demonstrated the photodynamic inactivation of ribonuclease by riboflavin using other assay procedures. Shugar shows the quantum yield for lysozyme inactivation to be 0.006, a value close to the values reported herein for trypsin. This constitutes the only report we know of for the quantum yield for the photodynamic inactivation of an enzyme.

In addition to the above results on molecules in solution, there is one report of the quantum requirement for a cellular level effect. Blum and Gilbert (54,55) have determined the quantum requirement for photodynamic hemolysis of rat erythrocytes using such dyes as rose bengal and erythrosin.

The values are in the range of approximately 10^{10} quanta absorbed per erythrocyte hemolyzed; these values differ, then, by many orders of magnitude from those for molecules in solution. Effects as a function of light intensity followed the reciprocity law and were essentially independent of dye concentration.

The examples listed above from the literature concerning quantum yield measurements in organic chemistry and experimental biology show the wide range of systems that have been studied and the kinds of values obtained. The study reported here involves a system that is more complicated than the photochemistry of simple organic molecules, and yet one that is very much simpler than the whole cell. Quantum yield measurements are an important aspect of any attempt to delineate mechanisms of reactions in photochemistry, since they establish the overall efficiencies of the reaction.

C. Photodynamic Sensitizing Dyes

A very large number of different dyes have been examined in a wide range of photodynamic systems. Blum (21) has reviewed the earlier work on the relation between dye structure and photodynamic ability. More recently, Fowlks (1) has tabulated the photodynamic activities of some 40 different dyes. This list includes certain hydrocarbons (such as 3,4-benzpyrene), dibenzopyran derivatives (such as eosin Y), phenoxazine derivatives (such as nile blue), phenazine derivatives (such as safranine), acridine derivatives (such as acriflavin), phenothiazine derivatives (such as methylene blue), furocoumarins (such as psoralen), furochromones

(such as visnagin), miscellaneous tricyclic compounds (such as riboflavin), porphyrin compounds (such as chlorophyll and hematoporphyrin), triphenyl methane compounds (such as methyl violet), and miscellaneous compounds (such as diethylstilbesterol and sulfanilamide). Oster (56) has examined the photodynamic activity of several dyes with respect to their ability to sensitize the photooxidation of p-toluenediamine. Those compounds found to be active include certain xanthenes, thiazoles, thiazines, acridines, porphyrins, and riboflavin. Those compounds found to be inactive include certain xanthenes, indamines, azo dyes, azine dyes, oxazines, phenyl methanes, anthraquinones, cyanines, acridines, nitro dyes, and indophenol.

Only certain of those categories of dyes listed above have been shown to sensitize enzyme photoinactivation. In this laboratory a number of different dyes have been shown to be active as sensitizers for the photodynamic inactivation of trypsin, e.g., certain acridines, two flavins (riboflavin and flavin mononucleotide, but not flavin adenine dinucleotide), some members of one sub-group of the azine dyes (the safranines), nearly all of the thiazine dyes examined, and most of the members of the phthalein subclass of the xanthene dyes. Hematoporphyrin, certain water-soluble chlorophyll derivatives, and a few simple substituted anthraquinones are also active. The inactive dyes with respect to the photodynamic inactivation of trypsin include the axo dyes, p-quinone imine dyes, the oxazine dyes, the triarylmethane dyes, the thiazole dyes, the ketone imine dyes, the methine dyes, the nitro dyes, and the nitroso dyes (15).

Three dyes were selected for the present study: methylene blue (MeB, a thiazine dye), eosin Y (a xanthene dye), and flavin mononucleotide (FMN, a flavin). FMN was chosen because it represents a unique sensitizer so far as is known (with the exception of riboflavin itself) in its ability to produce during photodynamic treatment a "damaged" class of trypsin molecule (a class of trypsin molecules which, although active, is very labile to such agents as heat or urea) (12,13); FMN also apparently inactivates trypsin by a different mechanism from all the other dyes so far tested in this laboratory.

MeB and eosin Y show somewhat the same kinetic behavior as sensitizers for the photoinactivation of trypsin; MeB was chosen because it has a positive charge, while eosin Y was chosen because it has a negative charge, in an effort to see if the charge on the dye molecule had any effect on its action as a sensitizer. The structures of the three dyes used are shown in Fig. 2.

A number of speculations have been made on the possible relationships between the physical-chemical properties of dye molecules and their photodynamic activity. The generalization has been made that only fluorescent dyes can sensitize, although the converse is certainly not true (21). A better generalization is that only "photoreducible" dyes (those able to form long-lived, probably triplet states) are photodynamically active (56). It has been shown in our laboratory, however, that dyes that are active in one system (i.e., trypsin photoinactivation) are not necessarily active in others (i.e., photodynamic alteration of rat-tail tendon).

Methylene Blue (a thiazine dye)
Absorption maxima = 668 mμ and 609 mμ

Eosin Y (a xanthene dye)
Absorption maximum = 515-518 mμ

Flavin Mononucleotide (a flavin dye)
Absorption maxima = 373 mμ and 445 mμ

Fig. 2. The structures and absorption maxima of the dyes used in this study for the dye-sensitized photodynamic inactivation of trypsin.

Dyes contain systems of conjugated double bonds with many possible resonance structures and hence many loosely bound π electron systems (57). These resonance forms are arranged so that light energy can be picked up by the molecule with the excitation of an electron to the singlet, followed by intersystem crossing to the long-lived (triplet) excited states in some cases. This energy can then be dissipated as fluorescent light or phosphorescent light when the excited molecule returns to the ground state. Incidentally, the fluorescent light and phosphorescent light were postulated by Viale (58) as being the photodynamically active light rather than the incident light, but this is no longer felt to be the case, and fluorescence and phosphorescence are now looked on as non-productive transitions back to the ground state. Alternatively, the energy of the excited electron can be used to perform chemical changes, as in Fig. 1.

In typical photodynamic systems, the dye is not irreversibly altered at an appreciable rate. Each dye molecule, therefore, can function many times in energy absorption and transfer. Under some reaction conditions, dyes are rapidly altered by photochemical processes. The photobleaching of riboflavin, eosin Y, and MeB is thought to be due to photoreduction (59-63). Ghiron (12) found that the photobleaching of dye was not a serious problem in his studies of the photodynamic inactivation of trypsin. Similarly, photobleaching has not been found to cause deviations from first-order kinetics in the work reported here.

Some proposed mechanisms for photodynamic action as illustrated in equation III of section A of this Introduction require a binding reaction of some type between dye and substrate (22). To what extent binding is required in photodynamic processes in general is not known. It is known

that photodynamically active dyes can bind to many different biological systems, as in the differential staining of subcellular constituents, for example. Ghiron (12) has shown that binding between MeB, eosin Y and FMN with trypsin, if it does occur, must be very weak. Spikes (14) has shown that binding of certain dyes to rat-tail tendon, with subsequent photo-inactivation in the form of altered mechanical properties, may be of a somewhat more permanent form. According to Harbury et al. (64), FMN-protein complexes can be demonstrated using spectral and kinetic evidence. In summary, it is probably fair to say that we don't know whether binding is a necessity for photodynamic action. According to most present theories, as mentioned in section A of this Introduction, some kind of dye-substrate interaction must occur in photodynamic action in order for energy to be transferred. The type of interaction remains obscure.

The foregoing discussion points out the fact that the mechanisms of action of photodynamic action are known at present only in a simple over-all way, and that much work must be done before a comprehensive understanding of them can be obtained.

D. The Biochemistry of Trypsin

Trypsin was chosen as the substrate for this study of dye-sensitized photoinactivation because it can be obtained in reasonably pure form and because there are several good assays for its enzymatic activity. A considerable amount of research has been done on the photodynamic inactivation of trypsin in this laboratory; further, its inactivation by ultraviolet and ionizing radiation has been investigated

extensively (65-67). An obvious disadvantage of this choice is that although the biochemistry of trypsin has been studied extensively (68,69), the actual amino acid sequence and molecular structure of trypsin have not been completely elucidated, although recent work has largely solved even this problem (70); trypsinogen consists of a linear sequence of 229 amino acid residues with six disulfide cross linkages. All 229 amino acid residues have been tentatively assigned positions in the linear sequence, but the locations of the six disulfide bonds are not certain.

Trypsin is a proteolytic endopeptidase which is secreted by the pancreas into the duodenum in an inactive form, trypsinogen. It is activated by enterokinase, certain mold kinases, and by trypsin itself autocatalytically. All three methods of activation give the same products. The activation is as follows:

The hexapeptide removed is Val(Asp)₄Lys, leaving the trypsin thus formed with a molecular weight of 23,800 according to the best available estimates (68). The rate of appearance of these hexapeptides and the rate of development of the enzymatic activity of the resulting trypsin correlate in an excellent manner (68). The peptide is apparently split off from the N-terminal end of the trypsinogen, leaving Ileu-Val-Gly as the N-terminal sequence in the trypsin itself. The free C-terminal end has recently been found in trypsinogen to be asparagine (70).

Trypsin catalyzes the splitting of peptide, amide, or ester linkages adjacent to lysine and arginine only. Trypsin will catalyze the splitting of native proteins only slightly; it works much faster on denatured proteins

(71). It will also catalyze the splitting of a variety of synthetic compounds. Benzoyl-L-arginine ethyl ester (BAEE) was chosen as the substrate in the present study.

Trypsinogen is obtained from acid extracts of fresh cattle pancreas and purified using ammonium sulphate fractionation (72). It is then activated as already described. The resulting trypsin appears to exist in the form of two or three different fractions as observed using electrophoretic methods of separation (73-75).

A number of studies have been carried out in attempts to elucidate the nature of the active site of trypsin. Such studies are directly pertinent to the present work, since ideally one would like to determine in precise detail how the active site is altered during photodynamic inactivation of the enzyme. Studies with diisopropylfluorophosphate, p-nitrophenylacetate, N-bromosuccinamide, etc., implicate serine in the active site of trypsin since they combine with this amino acid residue with resultant decrease in enzymatic activity (76,77). In addition, titration data give pK values which implicate histidine in the active site (78). The actual physical arrangement of the active site is unknown. Photodynamic oxidation of histidine and other amino acids has been shown to occur (9-11). Weil et al. (79-81), have been able to correlate the loss of enzymatic activity with the photooxidation of these amino acids in such enzymes as lysozyme, chymotrypsin and ribonuclease, presumably because the photooxidation alters the active site. This has been done more recently with lactic dehydrogenase (82). It is reasonable to assume that this occurs in trypsin as well.

Trypsin is most stable at pH 3.0 and hence was stored at this pH during the present work. It is enzymatically most active at pH 8.0, but at this pH it is also most active in self-digestion (autocatalytic). For this reason, all of the experiments reported here were carried out in time intervals (approximately) such that self-digestion was not a problem at the temperature used. Nevertheless, autocatalysis was checked for as a part of each series of experiments.

E. Inhibition of Photodynamic Action

Photodynamic action, as outlined in section A of this Introduction, is a complicated series of reactions involving light, dye, molecular oxygen and substrate. The mechanisms of action are only known in broad outline. One way to study the mechanism of a system is to cause a perturbation of the system and see how the resultant behavior differs from the usual behavior. In photodynamic action, such a perturbation can be introduced by, among other ways, adding inhibitors to the reaction system. Conceivably, these inhibitors could act in many ways; some examples would be:

- (a) prevention of excitation of the dye
- (b) singlet quenching (decrease the singlet lifetime)
- (c) triplet quenching (decrease the triplet lifetime)
- (d) competition with the substrate for oxidation
- (e) binding with the substrate or dye to prevent interaction
(called noncompetitive inhibition in enzyme kinetics).

In the present work, a series of inhibitors have been studied which presumably act by two of the mechanisms listed above, i.e., by quenching the triplet state of the dye, and by competing with the substrate (trypsin) for oxidation. The older work on the chemical inhibition of photodynamic action has been reviewed by Santamaria (22).

"Competitive" inhibition studies - For the purposes of the present work, we will designate those compounds which appear to inhibit photodynamic action by competing with substrate for oxidation as "competitive inhibitors." These competitive inhibitors, in a sense, may be looked upon as acting as "scavengers" in the system in a manner analogous to the competitive scavengers studied in ionizing radiation biology and chemistry (83).

Busck (84) apparently carried out the first work on inhibitors of this type. He showed that eosin-sensitized hemolysis is retarded by blood serum and egg albumin, but not by amino glycosides or gelatin. Schmidt and Norman (85) pointed out that these latter two compounds do not inhibit because they do not contain any aromatic amino acids. They went on to show that both free aromatic amino acids and proteins containing such amino acids were able to inhibit. In general, compounds with phenolic groups were inhibitory, whereas non-aromatic amino acids and sugars were ineffective as inhibitors of photodynamic action unless present in very high concentration (86-88). Sacharoff and Sachs (89) found photodynamic action to be inhibited by sodium sulfite, while Schmidt and Norman (90), and later Noack (91), found that many kinds of inorganic salts were inhibitory. Viale (58) enunciated a theory of fluorescence quenching to explain these results. It seems more reasonable

now to assume that effective inhibitors of the types listed above compete with the substrate for the energy of the photoactivated dye molecule (22).

More recent work has shown that tryptophan and tyrosine inhibit the photodynamic inactivation of lysozyme (52). Compounds such as cysteamine, cystamine, S-(2-aminoethyl)isothiuronium bromide hydrobromide (AET), glutathione, vitamin C, cystine, methionine, etc., have also proven effective as inhibitors (22,92-95).

Oster et al. (56), found a quantum yield of 3.0 for proflavin-sensitized photooxidation of p-toluenediamine, indicating a chain reaction. This photooxidation can be inhibited by adding glutathione, ascorbic acid, and cysteine, but once the added compound is destroyed, the photooxidation of p-toluenediamine continues. Glucose, allylthiourea, thiourea, ethylenediaminetetraacetic acid, potassium iodide, etc., were ineffective as inhibitors of this reaction, at least in concentrations lower than 2×10^{-3} M.

Yamamoto (96), in studies of the photoinactivation of T-series coliphage, found that inactivation could be prevented by the presence of certain dyes which were photodynamically ineffective. He suggested that a dye, in order to act photodynamically, must bind at specific sites on the coliphage. Inhibition by inactive dyes such as crystal violet could then result if the inactive dye combined with these sites, thus rendering them inaccessible to active dye molecules. At least in some cases, then, dye-binding may be necessary for inhibition.

Some inhibitors can be effective even in whole organisms. For example, Nishie and Keyl (97) showed that mice photosensitized with rose bengal could be protected from light injury to some extent by treatment

with certain thiols, phenosafranine, cysteamine, cysteine, AET, and DL-homocysteine. The chemical inhibition of photodynamic action, therefore, is not confined only to systems such as molecules and cells.

Triplet quenching studies - In general, energy transfer in photodynamic reactions appears to occur via the triplet state of the dye. The quenching of the triplet state of the sensitizing dye, therefore, represents another possible mechanism for the inhibition of photodynamic action.

Weber (98) has studied the fluorescence quenching of riboflavin and flavin adenine dinucleotide in great detail. The quenchers used and their relative efficiencies were: adenine (0.50); adenosine (0.50); hypoxanthine (0.51); caffeine (0.53); muscle adenylic acid (0.63); thymine (0.71); cytosine (0.77); allantoin (0.99); potassium iodide (0.72); hydroquinone (0.66); catechol (0.65); silver nitrate (0.03); silver acetate (0.03). Weber felt that the action of potassium iodide is to change the electronic excitation energy into kinetic energy. As the potassium iodide concentration increases, the quenching increases. Calculations show the riboflavin excited state lifetime to be about 1×10^{-8} sec. The mean lifetime is decreased by a factor of 7.7 in 0.6 M potassium iodide. The purines mentioned also alter the lifetimes, and a flavin-purine complex is suggested by the data. Livingston and Pugh (99,100) found many triplet quenchers for chlorophyll in organic solvents; some may be active in this system also.

These studies above all seem to indicate that dyes can be quenched when in the triplet state. Whether or not these same data apply to photodynamic systems is unknown.

Berends and Posthuma (29) found that in the photodynamic destruction of pimaricin with riboflavin and lumichrome as the sensitizers, various compounds had protective effects on pimaricin destruction and quenched riboflavin and lumichrome phosphorescence, which means that these compounds shorten the triplet lifetime. Potassium iodide, thiourea, thio-uracil, and ascorbic acid were particularly effective. Paramagnetic metal ions also exhibit these two effects. Nickel chloride, cupric chloride, manganese dichloride, and chromous chloride were particularly effective. A compound that quenches the phosphorescence of riboflavin supposedly acts by facilitating the radiationless dissipation of excitation energy by collisions with molecules of the solvent.

Oster and Adelman (63) show that in the eosin-sensitized photooxidation of allylthiourea, p-phenylenediamine hydrochloride reduces the reaction efficiency by half, probably by quenching the metastable state. Potassium iodide works also but not so effectively; they estimate that potassium iodide is only 1/100th as effective as p-phenylenediamine in quenching the metastable state of eosin.

If the triplet states of the excited dyes are involved in photodynamic action, this also suggests the formation of free radicals, since the promotion of an electron from the ground state to a triplet state implies a reversal of its electronic spin quantum number and hence of the production of an unpaired electron. Electron spin resonance studies have indicated the presence of free radicals in photodynamic dyes (22,101).

It has been shown that free radicals are produced during the photo-dynamic alteration of serum proteins as sensitized by hematoporphyrin; such radicals are produced only in the presence of oxygen (28). Flash photolysis techniques are being used to investigate the nature and the lifetimes of these free radicals and other excited states (102,8).

II. STATEMENT OF THE PROBLEM

Most living organisms and biological molecules are relatively insensitive to damage by visible light. Many biological systems, however, can be sensitized to visible light by certain dyes. Damage to biological systems by visible light in the presence of a sensitizing dye is known as "photodynamic action;" molecular oxygen is consumed in the process. This phenomenon has been shown to occur at all levels of biological complexity from isolated amino acids to human beings. The detailed mechanisms of photodynamic action are unknown because relatively little quantitative work has been done in this field. Quantum yields for photodynamic action represent one of the important kinds of quantitative data needed in this area in order to establish the mechanisms of inactivation.

This thesis represents an investigation of the quantum yields of the dye-sensitized photodynamic inactivation of the enzyme trypsin with respect to kinetic parameters and selected inhibitors. The kinetic parameters studied were dye concentration, enzyme concentration, buffer concentration, pH, temperature, ionic strength, light intensity, and stirring rate. The inhibitors used were sodium iodide, histidine, S-(2-aminoethyl)isothiuronium bromide hydrobromide (AET), and egg albumin.

Three different sensitizing dyes were used: methylene blue, eosin Y, and flavin mononucleotide. Quantum yield determinations allow direct comparisons to be made of the results with different dyes, since the quantum yield is the amount of inactivation per unit quanta, a dimensionless number. The results of these studies have been used to evaluate various mechanisms proposed for the process of photodynamic action. Photodynamic action does not occur by way of any of the simple kinetic schemes which have been proposed; further, the mechanism appears to be different with different photosensitizing dyes.

III. MATERIALS AND METHODS

A. Preparation of Reagents

Benzoyl-L-arginine ethyl ester (BAEE): This solution was prepared by dissolving 0.1 gm BAEE (Mann Research Laboratories No. 707) in 1 liter of 0.05 M sodium phosphate buffer at pH 8.0. The solution is stable for weeks when stored at 3° C.

Trypsin: A solution of 0.2 gm of 2 X crystallized salt-free trypsin (Worthington Biochemical Corporation Lot TRSF 823-24) in 30 ml of 0.01 M hydrochloric acid was prepared and then was diluted up to 100 ml with 0.01 M monosodium phosphate. This solution is 84 uM in trypsin if the molecular weight of trypsin is assumed to be 23,800. The final solution has a pH of 3 and is stable for months if stored at 3° C. Care was taken to prevent bubble formation in the solution during preparation since this could cause inactivation of the trypsin.

Phosphate buffer: Solutions of 0.5 M monosodium phosphate, 0.5 M disodium phosphate, 0.5 M trisodium phosphate, and 0.5 M phosphoric acid were made up of reagent grade chemicals and mixed in various amounts to secure solutions with pH values from 2.0 - 11.5. Measurements of pH were made with a Leeds and Northrup glass electrode pH meter.

Methylene blue (MeB): A 4×10^{-4} M solution of MeB (California Biochemicals No. 4605 zinc-free) was prepared, diluted as needed, and stored at room temperature in the dark.

Eosin Y: A 1×10^{-3} M solution of eosin Y (Allied Chemical Certificate NE37) was prepared, diluted as needed, and stored at room temperature in the dark.

Flavin mononucleotide (FMN): An 8×10^{-4} M solution of FMN (Nutritional Biochemicals Corporation) was prepared, diluted as needed, and stored at room temperature in the dark.

Inhibitors: The inhibitors studied were dissolved in the several dye solutions in such a way as to give the desired final concentrations after the components of the reaction system were mixed. Reagent grade sodium chloride and sodium iodide were used. The other inhibitors included reagent grade DL-histidine, reagent grade S-(2-aminoethyl)isothiuronium bromide hydrobromide (AET), and egg albumin (Mann Research Laboratories No. A4855, 5 X crystallized).

B. Experimental Procedures

Reaction mixture: The reaction cell contained a 0.25 ml aliquot of the following mixture: 1.0 ml of 84 μ M trypsin, 0.5 ml of 0.5 M sodium phosphate buffer at pH 8.0 unless otherwise specified, and 0.5 ml of standard dye solution, unless otherwise specified. The standard dye solutions were such as to provide final concentration of dye as follows: MeB, 12.5 μ M; eosin Y, 12.5 μ M; and FMN, 150 μ M.

Reaction conditions: Ten-microliter samples of the reaction mixture were taken before illumination and at 5-min intervals during the illumination and assayed for tryptic activity using the BAEE assay. This assay is described in detail in the next section. The inactivations were carried out at 16° C in air unless otherwise specified. The inactivations were always run at least in duplicate, and in some cases up to ten measurements were made; therefore, the quantum yields given are the

averages of these measurements. In experiments with MeB and eosin Y, the reaction mixtures were not stirred during illumination since preliminary experiments showed that stirring had no effect on the reaction rate. The FMN reaction mixtures were stirred since the inactivation deviated from first-order kinetics otherwise.

"Dark control" experiments were routinely run in which all of the reagents were added to the illumination cell and allowed to stand in the dark. These experiments demonstrated that the dyes in the concentrations used did not directly affect the trypsin, except at very high temperatures.

"Light control" experiments were also routinely run in which all of the reagents were added to the illumination cell except that the dye solution was replaced with distilled water. Illumination was carried out as usual. These experiments showed that there was no photochemical inactivation in the absence of dye.

C. Kinetics of the BAEE Assay for Trypsin Activity

Prior to the present study, the BAEE assay for trypsin activity had not been used to any great extent in this laboratory. Since the results of this study depended in such an important way on the assay for enzyme activity used, it was felt necessary to make a critical study of the kinetics of the assay itself. This section is devoted to the results of such a study.

BAEE is a synthetic substrate for trypsin; its hydrolysis is catalyzed by trypsin as shown in the following equation:

As the reaction proceeds, the optical density of the solution at 2530 Å increases in direct proportion to the amount of hydrolysis of BAEE because benzoylarginine absorbs maximally at this wavelength (103). The assay is conducted as follows: a 10- μ l aliquot of the dye-sensitized trypsin solution is mixed with 5 ml of the BAEE solution in a plastic vial. The optical density of this solution is measured with time in a quartz cuvette of 1 cm path length against BAEE solution using a Cary Model 14 recording spectrophotometer. The assay was routinely run at 30° C. Plastic vials were used for mixing because trypsin adsorbs to this material minimally. Fig. 3 shows a tracing of the recording of the optical density at 2530 Å of the BAEE solution as a function of time after native trypsin was added. As may be seen, the optical density increase is linear with time over the time period indicated. The change in optical density per 3 min time was arbitrarily chosen as our assay end point and as the value we would have represent the tryptic activity; the more active trypsin molecules added, the higher the value. Ten microliters of trypsin-dye solution in 5 ml of BAEE causes an inconsequential dilution factor in the BAEE. Also, the dye is too dilute after this dilution in the BAEE to cause any problem in the optical density measurements.

The effect of the amount of trypsin added on the BAEE reaction rate - trypsin catalyzes the hydrolysis of BAEE, and the reaction rate (change in optical density/3 min) increases in direct proportion to the amount of trypsin added. Fig. 4 shows the effect of the amount of trypsin added to the BAEE solution on the reaction rate at 2530 Å. As may be seen, the reaction rate increases linearly with the volume of 84 μ M trypsin added over the range of 2.5 - 100 μ l (representing 0.00021 - 0.0084 μ M of trypsin).

Fig. 3. The optical density change with time of the BAEE assay at 2530 Å. Ten microliters of 84 μ M trypsin were added to 5 ml of BAEE. The reaction was carried out at pH 8 and 30° C. This figure is a tracing of a graph produced by the Cary recording spectrophotometer.

Fig. 4. The effect of the amount of trypsin added on the BAEE reaction rate. The reactions were carried out at pH 8 and 30° C.

The effect of the pH of the BAEE solution on the BAEE reaction rate - The rate of hydrolysis of BAEE as catalyzed by trypsin is pH-dependent. The effect of pH on the reaction rate is shown in Fig. 5. As may be seen, the rate is very low at pH 5.9 and rises with increasing pH, passing through a maximum at approximately 8.0. On this basis, all assays, unless otherwise specified, were carried out at pH 8.

The effect of the pH of the trypsin solution on the BAEE reaction rate - The pH of the trypsin solution (over the range pH 2 - 8.5) added to the BAEE solution has only a small effect on the rate of the BAEE breakdown rate, as shown in Fig. 6. This result would be expected since the volume of the trypsin sample is so small.

D. The Illumination Apparatus

The illumination apparatus (Fig. 7) for the photodynamic inactivation of trypsin consisted of a turret arrangement plus a light source. A lucite reaction cell, with a constant temperature circulating water chamber immediately behind it, was mounted on one face of the turret. A vacuum thermocouple in a brass block was mounted behind the water chamber such that the reaction cell and the thermocouple were centered on the same optical axis. The amount of light passing through the reaction cell, and hence the amount of light absorbed in the cell, could thus be measured. The reaction cell was 0.6 cm thick by 0.7 cm high by 1.2 cm wide, with a volume of 0.5 ml. We routinely filled it only half full in order to permit better oxygen diffusion into the solution. This volume was adequate since the aliquots removed for assay were so small. Also,

Fig. 5. The effect of the pH of the BAEE solution on the BAEE reaction rate. The reactions were carried out at 30° C; the 10 microliter aliquots of trypsin added to the BAEE were at pH 8.

Fig. 6. The effect of the pH of the added trypsin solution on the BAEE reaction rate. The BAEE was at pH 8 and the reactions were carried out at 30° C.

Top View Showing Hg Arc Light

Side View Showing Standard Light Projector

Fig. 7. The illumination apparatus for dye-sensitized photodynamic inactivation of trypsin.

this volume of solution completely subtended the thermocouple-sensitive area with respect to the light source. The thermocouple was mounted in a brass block 2 cm thick by 6.5 cm high by 6.5 cm wide. This block provided adequate protection for the thermocouple and also tended to act as a heat sink for the dissipation of the thermally degraded photons.

On the face of the turret opposite from the reaction cell, a lucite setup was arranged to hold the thermocouple so that it was possible to measure the incident light impinging on the reaction cell.

The stirring apparatus consisted of a spring-loaded reciprocating stirrer operated by a motor-driven cam. The part of the shaft in contact with the solution was covered with polyethylene tubing. At 1800 cycles/min (our standard rate) the inactivation rate curve with respect to stirring rate is flat for the FMN-sensitized system. The trypsin is inactivated directly if the stirring rate is too great.

The cell was illuminated with light either by a 500-watt projector or by a high-pressure mercury arc (General Electric, AH-6, water-cooled) provided with multilayer interference filters (Baird Atomic Inc.). The following wavelengths were used for the different dyes as indicated: MeB, 6750 Å (band width at fifty per cent intensity $\leq$ 200 Å); eosin Y, 5170 Å (band width at fifty per cent intensity $\leq$ 200 Å); and FMN, 4370 Å (band width at fifty per cent intensity $\leq$ 200 Å). The reaction cell and the interference filters of the light sources were about 5 cm apart at maximum light intensity. Light intensity could be varied as desired by moving the light sources further away.

E. Measurements of Light Energy

In order to calculate quantum yields, both the amount of trypsin inactivated and the number of light quanta absorbed must be measured. Light energy absorbed was measured using a vacuum thermocouple (General Electric No. 91X458; internal resistance = 4 ohms; sensitivity, 1 microwatt/cm² = 0.08 microvolts) with the output fed into a galvanometer (Leeds and Northrup mirror type, No. 2285B, internal resistance = 16.7 ohms, period = 5.4 sec, sensitivity = 0.2 microvolts/mm). The thermocouple was wired in series to a decade resistance box (Heath Company Model DR-1) which allows many orders of magnitude of light intensity to be measured by adjusting the resistance of the circuit. Fig. 8 shows the light-detector wiring diagram. The thermocouple is wavelength-insensitive, and thus the system could be standardized directly, using a standard lamp (National Bureau of Standards, carbon filament type) (104). The response of the system was linear with light intensity. This was established using a rotating sector wheel flashing light setup. Fig. 9 shows the calibration curves. A secondary standard lamp was maintained for daily calibration of the system.

As mentioned above, the turret could be precisely positioned with respect to the light beam to permit measuring either the incident or the absorbed light. The following notations will be used in describing light measurements:

I_0 = the light passing through the illumination cell containing trypsin solution without dye.

Fig. 8. The wiring diagram for the thermocouple-galvanometer light-measuring system. The thermocouple responds to the light by putting out a current which is measured with a mirror-type galvanometer in series with decade resistances and a double-pole double-throw reversing switch. The decade resistances allow many orders of magnitude of light intensity to be measured, and the reversing switch allows the direction of movement of the mirror to be reversed.

Fig. 9. The calibration curve for the thermocouple-galvanometer light-measuring system. The curves show that the deflections are linear with light intensity. The lower abscissa is calibrated to obtain the incident light intensity corresponding to a given deflection. The resistance values are the values of the decade resistances.

I_s = the light passing through the illumination cell containing a trypsin-dye mixture.

$I_{abs} = I_o - I_s$ = the light absorbed in the trypsin-dye solution in the illumination cell.

I_{in} , the light incident in the cell itself, was measured with the turret turned so that the thermocouple-sensitive element was placed in the optical axis of the light beam in a position corresponding to that occupied by the center of the reaction cell during an inactivation experiment. The fraction of light absorbed was measured with the turret turned so that the reaction cell itself was in the light beam. The amount of light actually absorbed, I_{abs} , could then be found from the following equations:

$$\text{(Fraction of light absorbed)} = \frac{I_o - I_s}{I_o}$$

$$I_{abs} = I_{in} \text{ (Fraction of light absorbed)}$$

In practice, the deflections corresponding to I_o and I_s were used as such in the calculations without conversion to absolute units, and hence only one conversion (for I_{in}) was needed per experiment.

Fluorescent light correction - A possible source of error in measuring the light absorption of solutions of fluorescent materials such as photodynamically active dyes can result from the fluorescent light emitted by such solutions on illumination. This possibility in the present system was evaluated as follows: the distance from the center of the illumination cell to the thermocouple is 3.0 cm, and the sensitive area of the thermocouple is 0.36 cm^2 . The sphere subtended by this 3.0 cm distance is:

$$\text{Sphere area} = 4 \pi r^2 = 4(3.14)(3.0)^2 = 113 \text{ cm}^2$$

The total fluorescent light subtended by the thermocouple is thus the ratio:

$$\frac{\text{Sensitive area of thermocouple}}{\text{Sphere area}} = \frac{0.36}{113} = 0.0032$$

Thus, only 0.32% of the total fluorescent light is intercepted by the thermocouple. Assuming fifty per cent of the absorbed light is re-emitted as fluorescence, this would be at most 45×10^{-4} watts/cm²; 0.32% of this is 0.14×10^{-4} watts/cm². This value is so small (less than one per cent of the absorbed light) that fluorescent light corrections have been ignored.

Per cent error in the light intensity measurements - The absorbed light energy for the MeB-sensitized photodynamic action system was measured ten times as a check of reproducibility. The value obtained was 0.0086 ± 0.0002 watts/cm² at ninety-five per cent confidence limits, representing an error of 4.5%. This is only half the relative error found in the trypsin inactivation measurements and hence does not represent the major source of error in the calculated quantum yields.

F. Quantum Yield Equations Used With the Different Dyes

A set of standard equations was set up for the calculation of quantum yields from the experimental data since a very large number of such calculations were to be made. These greatly simplify the routine calculations.

The general equations are derived as follows: since

$$\Phi = \frac{\text{Number of trypsin molecules inactivated}}{\text{Number of quanta absorbed}}, \text{ or}$$

$$\Phi = \frac{(dS/dt)_0}{(dQ/dt)_0}, \text{ where } S = \text{substrate (trypsin) and } Q = \text{quanta, or}$$

$$\Phi = \frac{(E_0/t_{37})}{(dQ/dt)_0}$$

where E_o = initial trypsin concentration, and t_{37} = time when only thirty-seven per cent of the activity remains, as discussed in section A of the Results.

Under our conditions, the initial trypsin concentration is

$$E_o = 42 \times 10^{-6} \frac{\text{moles}}{\text{liter}} \times .0025 \text{ liters} = 0.0105 \times 10^{-6} \text{ moles}$$

therefore,

$$E_o = 0.0105 \times 10^{-6} \text{ moles} \times 6.023 \times 10^{23} \frac{\text{molecules}}{\text{mole}} = 63.25 \times 10^{14} \text{ molecules.}$$

The energy per quantum is given by

$$E = \frac{hc}{\lambda} = 6.625 \times 10^{-34} \text{ Joule sec} \frac{3 \times 10^{10} \text{ cm/sec}}{\lambda(\text{in cm})}$$

For MeB at 6750 Å:

$$E = 6.625 \times 10^{-34} \text{ Joule sec} \frac{3 \times 10^{10} \text{ cm/sec}}{6750 \times 10^{-8} \text{ cm}} = 29.4 \times 10^{-20} \frac{\text{Joules}}{\text{quanta}}$$

For eosin Y at 5170 Å:

$$E = 6.625 \times 10^{-34} \text{ Joule sec} \frac{3 \times 10^{10} \text{ cm/sec}}{5170 \times 10^{-8} \text{ cm}} = 38.5 \times 10^{-20} \frac{\text{Joules}}{\text{quanta}}$$

For FMN at 4370 Å:

$$E = 6.625 \times 10^{-34} \text{ Joule sec} \frac{3 \times 10^{10} \text{ cm/sec}}{4370 \times 10^{-8} \text{ cm}} = 45.5 \times 10^{-20} \frac{\text{Joules}}{\text{quanta}}$$

$$(dQ/dt)_o = I_{\text{abs}} \frac{\text{watts}}{\text{cm}^2} \times 0.42 \text{ cm}^2 \text{ of front surface when cell contains 0.25 ml} = 0.42 I_{\text{abs}} \text{ watts} = 0.42 I_{\text{abs}} \frac{\text{Joules}}{\text{sec}}$$

For MeB at 6750 Å:

$$(dQ/dt)_o = 0.42 I_{\text{abs}} \frac{\text{Joules}}{\text{sec}} \times \frac{1 \text{ quanta}}{29.4 \times 10^{-20} \text{ Joules}} = \frac{0.42 I_{\text{abs}}}{29.4 \times 10^{-20}} \frac{\text{Quanta}}{\text{sec}}$$

For eosin Y at 5170 Å:

$$(dQ/dt)_o = 0.42 I_{\text{abs}} \frac{\text{Joules}}{\text{sec}} \times \frac{1 \text{ quanta}}{38.5 \times 10^{-20} \text{ Joules}} = \frac{0.42 I_{\text{abs}}}{38.5 \times 10^{-20}} \frac{\text{Quanta}}{\text{sec}}$$

For FMN at 4370 Å:

$$(dQ/dt)_0 = 0.42 I_{abs} \frac{\text{Joules}}{\text{sec}} \times \frac{1 \text{ quanta}}{45.5 \times 10^{-20} \text{ Joules}} = \frac{0.42 I_{abs}}{45.5 \times 10^{-20}} \frac{\text{Quanta}}{\text{sec}}$$

The quantum yields then are (with t_{37} in min and I_{abs} in watts/cm²):

For MeB at 6750 Å:

$$\Phi = \frac{E_0/t_{37}}{(dQ/dt)_0} = \frac{\frac{63.25 \times 10^{14} \text{ molecules}}{t_{37} \text{ min (60 sec/1 min)}}}{\frac{0.42 I_{abs}}{29.4 \times 10^{-20}} \frac{\text{Quanta}}{\text{sec}}} = \frac{73.8 \times 10^{-6}}{t_{37} I_{abs}}$$

For eosin Y at 5170 Å:

$$\Phi = \frac{\frac{63.25 \times 10^{14} \text{ molecules}}{t_{37} \text{ min (60 sec/1 min)}}}{\frac{0.42 I_{abs}}{38.5 \times 10^{-20}} \frac{\text{Quanta}}{\text{sec}}} = \frac{96.3 \times 10^{-6}}{t_{37} I_{abs}}$$

For FMN at 4370 Å:

$$\Phi = \frac{\frac{63.25 \times 10^{14} \text{ molecules}}{t_{37} \text{ min (60 sec/1 min)}}}{\frac{0.42 I_{abs}}{45.5 \times 10^{-20}} \frac{\text{Quanta}}{\text{sec}}} = \frac{114.2 \times 10^{-6}}{t_{37} I_{abs}}$$

These equations were used in computing the value of Φ ; t_{37} is measured in min and varied from ~3 min to ~100 min under these conditions. I_{abs} is measured in watts/cm² and under these conditions varied from ~10 x 10⁻⁴ watts/cm² to ~100 x 10⁻⁴ watts/cm².

IV. RESULTS

A. Introductory Experiments on the Photodynamic Inactivation of Trypsin

Time course of inactivation - The time course of the photodynamic inactivation of trypsin under our conditions can be fitted to first-order kinetics. This is shown in Fig. 10, where the log of the relative trypsin activity remaining (expressed as the change in optical density/3 min of the BAEE assay reaction) is plotted as a function of the illumination time. The mathematical expression for this relationship is as follows:

$$\frac{(\Delta \text{OD}/3 \text{ min})}{(\Delta \text{OD}/3 \text{ min})_0} = E/E_0 = e^{-kt}$$

where E is the enzyme activity remaining at any time, t, E_0 is the initial enzyme activity, and k is the first-order reaction rate constant in min^{-1} . When $kt = 1$, then $E/E_0 = e^{-1} = 0.368 \approx 0.37$. Thus, the " t_{37} value," i.e., the time at which thirty-seven per cent of the enzyme activity remains, is equal to $1/k$ and hence, by determining t_{37} , we can calculate the first-order reaction rate constant. The t_{37} value also is useful since this represents one of the easiest ways to arrive at the initial velocity, v_0 , of the reaction, a value that is needed to calculate the quantum yield. This relationship was shown by Lea (105) to be: $v_0 = E_0/t_{37}$. A graphical illustration of this relationship is shown in Fig. 11.

The effect of the assay temperature on the apparent trypsin inactivation rate - The trypsin photoinactivation was carried out at 16°C , while the BAEE assay for trypsin activity was carried out at 30°C . Experiments were carried out to see if this temperature change made any difference in the apparent photodynamic inactivation rate of trypsin since the assay

Fig. 10. The first-order inactivation rate curve for the photodynamic inactivation of trypsin. The reaction was carried out with 12.5 μM MeB in 42 μM trypsin at pH 8 and 16° C. The buffer was 0.125 M sodium phosphate.

Fig. 11. The first-order inactivation rate curve for the photodynamic inactivation of trypsin. This rectilinear plot shows graphically that $v_o = E_o / t_{37}$. The reaction system was 12.5 μM in MeB, 42 μM in trypsin, and 0.125 M in sodium phosphate buffer at pH 8 and 16° C.

temperature has been shown to affect the apparent quantum yield for the ultraviolet radiation inactivation of chymotrypsin (106). In the present case, the temperature during the assay was varied over the range 5-39° C without producing any apparent differences in the rate of the photodynamic inactivation of trypsin (Fig. 12). At higher assay temperatures, the absolute magnitudes of inactivation change, but not the rate of inactivation (the slope of the line). It would appear, therefore, that no further selective inactivation is produced by carrying out the BAEE assay at 30° C.

Reproducibility of the trypsin inactivation measurements - Ten separate measurements of the t_{37} value of photoinactivation of trypsin by MeB under standard conditions were carried out. The average t_{37} value was 6.02 ± 0.26 min at ninety-five per cent confidence limits. This corresponds to an error in our measurements of inactivation of eight per cent. This is our major source of error since the light intensity measurements are more precise.

Reproducibility of the quantum yield measurements - As shown above for a MeB-sensitized system, the error in the determination of t_{37} was eight per cent at the ninety-five per cent confidence limits; as described earlier, the error in the light measurements was 4.5% at the ninety-five per cent confidence limits. A series of ten quantum yield measurements on MeB-sensitized photodynamic action gave a value of 0.00143 ± 0.00007 at the ninety-five per cent confidence limits. The overall error in quantum yield measurements is therefore nine per cent. The t_{37} values could probably be measured a little more precisely by using a Beckman DU spectrophotometer rather than the Cary instrument. Further, instruments such as a Keithley millimicrovoltmeter could be used for measuring light energies.

Fig. 12. The effect of the temperature of the BAEE assay solution on the trypsin photoinactivation rate. The reaction systems were 12.5 μ M in MeB, 42 μ M in trypsin, and 0.125 M in sodium phosphate buffer at pH 8 and 16°C.

Thus, the error in quantum yield measurements could probably be reduced to some extent; this was not felt necessary for the present studies.

B. Effect of Stirring on the Photodynamic Inactivation of Trypsin

It is well known that stirring can influence the rates of reactions greatly due to increased mixing. In the present case, since molecular oxygen is involved in the photodynamic inactivation of trypsin, stirring will greatly facilitate the diffusion of oxygen from the air into the solution. Experiments were therefore carried out on our system to determine the effect of stirring on the photoinactivation rate of trypsin. Stirring was carried out with a spring-loaded stirrer that was driven by an eccentric cam. The speed was adjusted (1800 strokes/min) so that no inactivation of trypsin occurred due to mechanical agitation. The data in Fig. 13 show that stirring had no effect on the rate of the photodynamic inactivation of trypsin in MeB- and eosin Y-sensitized systems. In the FMN-sensitized system, stirring had a profound effect. In the unstirred system, trypsin inactivation was very slow, and the reaction was no longer first-order. In all subsequent experiments, the eosin Y- and MeB-sensitized systems were not stirred, while the FMN-sensitized systems were stirred as described. First-order inactivations were always obtained in this way.

Fig. 13. The trypsin inactivation rate as a function of the stirring rate of the solutions. Stirring was carried out at 1800 reciprocations per min. The reaction systems were $42 \mu\text{M}$ in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 16°C , with either $12.5 \mu\text{M}$ MeB or eosin Y, or $150 \mu\text{M}$ FMN.

C. Quantum Yield as A Function of Dye Concentration

The effects of dye concentration on the quantum yield of the photo-dynamic inactivation of trypsin were examined. The results of a typical set of experiments are shown in Fig. 14. As may be seen, the relation between quantum yield and dye concentration is somewhat different with the different dyes. As the concentration of eosin Y increases, the quantum yield rises sharply and attains a maximum of 0.0027 at a concentration of 25 μM . Further increases in dye concentration result in a lowering of the quantum yield. As the concentration of MeB increases, the quantum yield rises sharply and reaches a plateau at a concentration of 12.5 μM and then gradually declines. As the concentration of FMN increases, the quantum yield increases slowly to a maximum at a concentration of 150 μM . On the basis of these studies, the following standard dye concentrations were chosen for further work: MeB, 12.5 μM ; eosin Y, 12.5 μM ; and FMN, 150 μM . It is difficult to compare these three cases directly because the quantum yield curves as a function of dye concentration are very different in shape in each case. Fig. 15 shows that the optical density of the illuminated system as a function of dye concentration is linear for the FMN system; the MeB and eosin Y systems are non-linear as measured in the reaction cell.

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Fig. 15. Optical density of the illuminated systems as a function of dye concentration.

D. Quantum Yield as A Function of Light Intensity

The quantum yield as a function of the rate of light energy absorption has been investigated. Typical results are shown in Fig. 16 plotted as reaction rate versus rate of light absorption. As can be seen, the reaction rates are linear as a function of the rate of light absorption. The actual slopes of the curves are not important since the dye concentrations used are much different: 12.5 μM for MeB and eosin Y, and 150 μM for FMN. Since the quantum yield is essentially the ordinate divided by the abscissa, the quantum yields would be expected to be independent of the rate of light absorption. This is true, as is shown by the data in Fig. 17.

E. Quantum Yield as A Function of Trypsin Concentration

The quantum yield was examined as a function of the trypsin concentration. Typical results are shown in Fig. 18. As can be seen, the quantum yield increases linearly with decreasing enzyme concentration with all three dyes as sensitizers. Trypsin over the range of 5 - 42 μM could be investigated conveniently with the technique described.

Similar sets of data were obtained at lower dye concentrations, i.e., in 6.25 and 3.13 μM MeB- and eosin Y-sensitized systems and in 50 and 20 μM FMN-sensitized systems. The curves at lower dye concentrations were similar in slope but of different absolute magnitudes since the amount of dye available for light trapping was smaller.

Fig. 16. The photodynamic inactivation rate of trypsin as a function of the rate of light absorption during illumination as varied by changing the incident light intensity. The reaction systems were 42 μ M in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 16° C. The dyes used were 12.5 μ M MeB or eosin Y, or 150 μ M FMN.

Fig. 17. The quantum yield of the photodynamic inactivation of trypsin as a function of the rate of light absorption during illumination as varied by changing the initial light intensity. The reaction systems were 42 μ M in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 16° C. The dyes used were 12.5 μ M MeB or eosin Y, or 150 μ M FMN.

Fig. 18. The quantum yield of photodynamic inactivation of trypsin as a function of the trypsin concentration during illumination. The reaction systems were 0.125 M in sodium phosphate buffer at pH 8 and 16 $^{\circ}$ C. The dyes used were 12.5 μM MeB or eosin Y, or 150 μM FMN.

F. Quantum Yield as A Function of pH

The rates of many photobiological and photochemical systems are dependent on pH. Fig. 19 shows the effects of pH on the photodynamic inactivation of trypsin. As can be seen, there is no photoinactivation in the MeB- and eosin Y-sensitized systems from pH 2 - 6. The quantum yields then rise sharply as the pH is further increased and level off in strongly basic solutions. In contrast, the FMN-sensitized photoinactivation of trypsin shows measurable quantum yields from pH 2 - 7. As with the other dyes, there is then a rapid increase in values on the basic side of the pH scale.

The range of pH values examined corresponds to the useful range which can be obtained using phosphate buffers. It was desirable to use the same buffer system throughout so that any differences found would not be due to different buffers. At high pH values (11 and above), rapid self-digestion of trypsin occurs; thus this range was not investigated. Self-digestion is not a problem over the pH range included here because the time-course of trypsin inactivation was kept short (usually less than 15 min).

G. Quantum Yield as A Function of the Sodium Phosphate Buffer Concentration

The quantum yield for trypsin photoinactivation with all three dyes was examined as a function of the sodium phosphate buffer concentration over the range 0.00125 - 0.125 M. Fig. 20 shows that the buffer concentration over this range does not affect the quantum yield. The pH was

Fig. 19. The quantum yield of the photodynamic inactivation of trypsin as a function of the pH during illumination. The reaction systems were 42 μM in trypsin at 16° C; the dyes used were 12.5 μM MeB or eosin Y, or 150 μM FMN. The buffers used were 0.125 M sodium phosphate buffer made up to cover the range of pH from 2 to 10.

Fig. 20. The quantum yield of the photodynamic inactivation of trypsin as a function of the phosphate buffer concentration during illumination. The reaction systems were 42 μM in trypsin and 12.5 μM in MeB or eosin Y, or 150 μM in FMN. The reactions were run at 16° C with the sodium phosphate buffer concentration varied but the pH held constant at 8.0.

maintained at 8.0 throughout regardless of the buffer concentration, and this was measured both before and after the trypsin inactivation.

H. Quantum Yield as A Function of Sodium Chloride Concentration

The effect of ionic strength on the quantum yield for trypsin photo-inactivation was examined using sodium chloride. Fig. 21 shows that sodium chloride concentration over the range 0 - 1.0 M has very little effect on the quantum yields of the FMN- and eosin Y-sensitized photoinactivation of trypsin. Sodium chloride alters the quantum yield of the MeB-sensitized photodynamic inactivation of trypsin drastically; however, the quantum yield decreases as the salt concentration increases. This phenomenon results from the fact that MeB precipitates out of solution at higher salt concentrations, presumably as a result of a common ion (Cl^-) effect.

In order to examine the effect of sodium chloride on these dye-sensitized systems, the salt was added to the dye solution and allowed to stand overnight at room temperature. The absorption spectrum of the mixture was then examined to see if any change in the dye concentration had taken place. The quantum yield measurements were then made. The decreased quantum yield could be accounted for in the MeB case on the basis of decreased dye concentration.

Fig. 21. The quantum yield of the photodynamic inactivation of trypsin as a function of the sodium chloride concentration during illumination. The reaction systems were 42 μ M in trypsin, 0.125 M in sodium phosphate buffer at pH 8 and 16°C with 12.5 μ M MeB or eosin Y, or 150 μ M FMN.

I. Quantum Yield as A Function of Temperature

Temperature alters chemical reaction rates markedly but has little effect on pure photochemical processes. The effect of temperature on the photodynamic inactivation of trypsin was examined, therefore, to determine whether the relative participation of photochemical and thermochemical processes in the inactivation mechanism could be assessed. The results of typical experiments are shown in Fig. 22. As may be seen, the quantum yields increase linearly with temperature over the range studied ($4 - 33^{\circ}\text{C}$) with all three dyes. The temperatures within the reaction cell were checked during illumination by using a calibrated microthermister and were found to vary less than 1.5°C during the illumination procedure.

These data can also be plotted in the form: reaction rate versus the reciprocal of the absolute temperature (Arrhenius plot) as shown in Fig. 23. The experimental activation energy, E_a , can be calculated from the slopes of the resulting curves. Typical values obtained with the different dyes are listed below.

150 μM FMN, $E_a = 2.8$ kcal/mole

12.5 μM eosin Y, $E_a = 3.7$ kcal/mole

12.5 μM MeB, $E_a = 4.8$ kcal/mole

The effects of temperatures below 4°C were not studied since the quantum yields are very small, while at temperatures above about 28°C , errors due to the self-digestion of trypsin become large. In these experiments, corrections for self-digestion were made at 28°C and at 33°C .

Fig. 22. The quantum yield of the photodynamic inactivation of trypsin as a function of the temperature during illumination. The reaction systems were $42 \mu\text{M}$ in trypsin, 0.125 M in sodium phosphate buffer at pH 8 and 16°C with $12.5 \mu\text{M}$ MeB or eosin Y, or $150 \mu\text{M}$ FMN.

Fig. 23. The rate of trypsin photodynamic inactivation as a function of the reciprocal of the temperature (Arrhenius plot). The slopes of the lines represent $E_a/2.303 R$. The experimental activation energies, the E_a values, are listed for each sensitizer. The reaction systems were 42 μ M in trypsin, 0.125 M in sodium phosphate buffer at pH 8 and 16° C with 12.5 μ M MeB or eosin Y, or 150 μ M FMN.

J. Quantum Yield as A Function of Sodium Iodide Concentration

Useful information can be obtained about photodynamic systems by studying the kinetics of the chemical inhibition of the reaction. The quantum yields of trypsin photoinactivation were studied with respect to sodium iodide concentration since this substance is thought to quench the triplet state of the dye (29,98). Fig. 24 shows that the MeB- and eosin Y-sensitized photodynamic inactivation of trypsin was inhibited by sodium iodide, although only at rather high concentrations. In contrast, the FMN-sensitized photodynamic inactivation of trypsin was inhibited strongly at very low concentrations of sodium iodide. The effect of sodium iodide on the sensitizers was examined by adding it to the standard dye solutions and allowing the mixtures to stand overnight at room temperature. The absorption spectra showed that sodium iodide acted by precipitating MeB out of solution (common halide ion effect); there was no precipitation of FMN or eosin Y.

K. Quantum Yield as A Function of Histidine Concentration

Histidine is capable of being photooxidized (9-11) and hence could act as a competitive inhibitor of the photodynamic inactivation of trypsin. The histidine was added to the standard dye solution as before. The mixture was then allowed to stand overnight at room temperature, and its absorption spectra determined. No dye was precipitated out in any case under these conditions. The accompanying graph (Fig. 25) shows that

Fig. 24. The quantum yield of the photodynamic inactivation of trypsin as a function of the sodium iodide concentration during illumination. The reaction systems were 42 μM in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 16° C with 12.5 μM MeB or eosin Y, or 150 μM FMN.

Fig. 25. The quantum yield of the photodynamic inactivation of trypsin as a function of the histidine concentration during illumination. Note the discontinuity in the scale on the abscissa. The reaction systems were 42 μM in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 16° C with 12.5 μM MeB or eosin Y, or 150 μM FMN.

histidine inhibits trypsin photodynamic inactivation as sensitized by all three dyes used, the curves being nearly linear with histidine concentration. The slope of the quantum yield versus histidine concentration is very steep with eosin Y as sensitizer, and very flat with FMN.

L. Quantum Yield as A Function of the AET Concentration

AET has been shown to protect a variety of biological systems to some extent against ionizing radiation (94,95). AET was added to the standard dye solutions. The mixtures were allowed to stand overnight at room temperature and their absorption spectra determined. AET, in the concentrations used, had no effect on MeB and FMN but did precipitate out some dye from eosin Y solutions. The effects of AET on the photodynamic inactivation of trypsin are shown in Fig. 26. AET acts as a protector with MeB and FMN as sensitizers. It appears that AET does not act to any great extent as a protector for the eosin Y-sensitized system, except by causing dye precipitation.

M. Quantum Yield as A Function of Egg Albumin Concentration

Egg albumin was examined as a protective agent in the photodynamic inactivation of trypsin. Crystalline egg albumin was added to the standard dye solutions. The mixtures were allowed to stand overnight in the cold and their absorption spectra determined. The egg albumin did not precipitate out any of the dyes used.

Fig. 26. The quantum yield of the photodynamic inactivation of trypsin as a function of the AET concentration during illumination. Note the discontinuity in the scale on the abscissa. The reaction systems were 42 μM in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 16° C with 12.5 μM MeB or eosin Y, or 150 μM FMN.

Fig. 27 shows that egg albumin protected trypsin against photodynamic inactivation. It protects the eosin Y-sensitized photodynamic inactivation of trypsin the best and FMN-sensitized photodynamic inactivation of trypsin the least.

Fig. 27. The quantum yield of the photodynamic inactivation of trypsin as a function of the egg albumin concentration during illumination. The reaction systems were $42 \mu\text{M}$ in trypsin and 0.125 M in sodium phosphate buffer at pH 8 and 16°C with $12.5 \mu\text{M}$ MeB or eosin Y, or $150 \mu\text{M}$ FMN.

V. DISCUSSION

The sections in this Discussion are in the same order as the data appear in the sections on Results. Each section will be discussed separately first, and then an attempt will be made to correlate this body of data and integrate it with the literature discussed.

A. Interpretation of the Effect of Stirring on Trypsin Photoinactivation

Under the experimental conditions used in this investigation, MeB- and eosin Y-sensitized photoinactivation of trypsin show no differences between stirred and non-stirred solutions with respect to the reaction rate, the inactivation proceeding rapidly. Also, the time-course of inactivation is first-order in all cases. In contrast, the reaction rate of the FMN-sensitized system when not stirred is slow, and the time-course of the inactivation deviates from first-order under these experimental conditions. The inactivation in the FMN-sensitized system is rapid and follows first-order kinetics when the solution is stirred. Experiments showed that the stirring as such did not inactivate the trypsin.

Since oxygen is used up in photodynamic action, the above observations are interpreted to mean that oxygen can diffuse into the MeB- and eosin Y-sensitized solutions sufficiently fast for rapid inactivation even without stirring, while for the FMN-sensitized solution, diffusion is too slow and oxygen becomes rate-limiting. In support for this conclusion is the fact that Ghiron (12) has shown that the ratio of molecules of oxygen used per trypsin molecule inactivated is 59 for the MeB-sensitized system and 393

for the FMN-sensitized system. Spikes (107) has obtained ratios of 5 for the MeB-sensitized system and 15 for the FMN-sensitized system under different conditions. It appears, then, that the FMN-sensitized photoinactivation of trypsin requires much more oxygen per trypsin molecule inactivated than the other two systems. Hence, in unstirred FMN-sensitized systems, this could lead to very slow reaction rates that would not follow first-order kinetics. Also, since the deviation from first-order kinetics occurs so soon after the start of illumination in the FMN-sensitized system, it would appear that the dependence of the quantum yield on oxygen concentration might be very different with the different dyes, with the MeB and eosin Y system curves falling slowly at low oxygen concentrations, and the FMN system curve falling sharply at an oxygen concentration near twenty-one per cent in the gas phase. Gaffron (48) found that chlorophyll-sensitized photodynamic systems were influenced only slightly by oxygen concentration. Millar and Schwert (82) found that oxygen concentration exerted an effect on the time-course of the photodynamic inactivation of lactic dehydrogenase as measured using an oxygen electrode; the reaction deviated from first-order kinetics at low oxygen concentration, with the rate of loss of activity progressively decreasing as the oxygen concentration decreased.

B. Interpretation of the Effect of Dye Concentration on Trypsin

Photoinactivation

The relationships between quantum yield and dye concentration (Fig. 14) reveal that no simple equations will fit the data. At 12.5 μM dye concentration, for example, MeB-, eosin Y-, and FMN-sensitized systems show the following relative quantum efficiencies: MeB, 1.00; eosin Y, 1.73; FMN, 0.68. In contrast, at 50 μM dye concentration the relative efficiencies have shifted to: MeB, 1.00; eosin Y, 1.28; and FMN, 1.24. Under optimum conditions of concentration, eosin Y is the most efficient of the three sensitizers, giving a quantum yield of 0.0027 at 25 μM ; the quantum yield at higher concentrations drops rapidly, perhaps due to concentration quenching. MeB-sensitized systems do not show such a drop until 100 μM , while FMN-sensitized systems increase all the way up to 150 μM before they decline. This is clear evidence for different mechanisms operating in the three cases because the quantum yields in each case show a different dependence on dye concentration. In photodynamic action at a given dye concentration, two dyes with identical molar extinction coefficients and mechanisms of action would give the same quantum yields, and moreover, their curves of quantum yield with respect to dye concentration would be identical. If their molar extinction coefficients were dissimilar but their mechanism of action identical, they would be related by a definite ratio of quantum yields at all dye concentrations, and their curves with respect to dye concentration would still be similar.

Chiron also indicated that the mechanisms of action of MeB, eosin Y, and FMN are different (12), based on the differences in their individual kinetic properties with respect to trypsin photoinactivation.

Karg (108) found that the quantum yield of the photodehalogenation of xanthene dyes in the presence of compounds containing tertiary nitrogen atoms increased as the dye concentration increased. He interpreted this to mean that the transition between the singlet excited state of the dye and the metastable excited state is induced by a ground state dye molecule. Increasing the number of ground state dye molecules would thus increase the intersystem crossing and hence the quantum efficiency.

In our trypsin system a similar increase in the quantum yield with increasing dye concentration is observed at low dye concentrations. Karg's interpretation above seems like a reasonable explanation for our results at low dye concentration. At high dye concentrations, however, the quantum yield of trypsin photoinactivation decreases. This latter effect could be due to the light being very efficiently trapped in the first few layers (dx) of the solution such that the remainder of the solution is relatively unexposed. This has been observed visually in our solution at high dye concentrations and may explain the lowered quantum yield at high dye concentrations, although other things such as concentration quenching may be operating. It has been shown that in MeB solutions at high dye concentrations, appreciable amounts of dimers form at approximately the concentration where the quantum efficiency decreases (109). MeB dimers do not fluoresce and hence may not be useful in photodynamic action, since all photodynamic dyes are fluorescent (21). McRae and Kasha (110) show, however, that some dyes increase in their phosphorescent

ability upon aggregation; such dyes might show increased photodynamic ability in the dimer form.

C. Interpretation of the Effect of Light Intensity on Trypsin
Photoinactivation

Within the light energies available in this study, the rate of the photodynamic inactivation of trypsin increases linearly with increasing light energy absorbed with all three dyes (see Fig. 16). Since the quantum yield is essentially the reaction rate divided by the quanta absorption rate, this shows that the quantum yield is constant over our light intensity range. This was verified by our calculations (Fig. 17). That is, the number of quanta absorbed per unit time has no bearing on their efficiency of utilization. At much higher incident light energies (as obtained with a laser), it might be possible to "light-saturate" photodynamic systems in a manner analogous to that observed in photosynthesis (111) and the Hill reaction (112). In these latter processes, light saturation occurs because of the limit of enzymes available for the dark reactions. The dye-sensitized photoinactivation of trypsin is not a pure photochemical process (as shown in this study by the positive E_a values obtained) and hence light saturation possibly could occur in a manner somewhat analogous to photosynthetic systems. On the other hand, considering just the photoactivation of the dye, the fact that the efficiency of utilization of the quanta is the same regardless of the number of quanta absorbed per unit time over the range investigated in this work could indicate that there are always sufficient dye molecules in

the ground state to pick up the light because the lifetimes of the excited states are so short. For 12.5 μ M MeB there are 1.9×10^{15} molecules of dye in the cell. With the highest incident light energy at our disposal at 675 m μ (160×10^{-4} watts/cm²), we have 2.3×10^{17} quanta/sec. This means that each molecule would only have to be excited slightly more than once in every 1/100 sec to account for absorption of all the incident photons. However, only fifty-five per cent of the incident photons were absorbed under these conditions, and hence to account for this absorption of photons, each dye molecule would only have to be excited less than once in every 1/100 sec. The calculations are similar for the other dyes used. It may be that the dyes in the excited states absorb at different wavelengths from the ground state.

By flashing light techniques (113), the lifetimes of the longest-lived excited species effective in photodynamic inactivation of trypsin in the MeB-sensitized system have been shown to be in the neighborhood of 0.001 sec under certain conditions, and hence we would have to increase our light energy by at least a factor of ten to cause light saturation in this system, assuming the per cent fluorescence would be the same at higher light intensities.

Light saturation could possibly be obtained also by decreasing the dye concentration. Decreasing the dye concentration in our studies is not too practical because the inactivation rate becomes too long for convenient assay and less light is trapped. It is reasonable to assume that light saturation could be shown if intensities of light great enough could be obtained because then the lifetimes of the excited dye molecules would be rate-limiting. Since the reaction rate with respect to light energy

absorbed is linear, this also indicates that under these conditions the photochemistry (absorption of light by the dye) is not rate-limiting.

The dye is permanently photoreduced only slightly, if at all, as the trypsin inactivation reaction proceeds. If the reaction could be run at higher light intensities, it probably would deviate from first-order kinetics as the dye is used up or destroyed in other kinds of reactions. Under the experimental conditions used here, enough active dye apparently is available at all times so that first-order kinetics are followed and the reaction is over before the dye has been altered permanently. Most of the activated species lose their energy by non-productive means (non-radiative transitions, fluorescence, phosphorescence, or other kinds of chemistry not involved in our measurements), and only one in about 1,000 ($\Phi \approx 0.001$) carries out any trypsin inactivation.

D. Interpretation of the Effect of Trypsin Concentration on Trypsin Photoinactivation

The quantum yield of the photodynamic inactivation of trypsin increases with decreasing enzyme concentration (Fig. 18). This is surprising because usually the opposite is found in photochemistry; the opposite situation could be interpreted to mean that at higher trypsin concentrations the chances for an activated dye molecule or dye-oxy-radical to interact with trypsin are greater since there are relatively more trypsin molecules per activated dye molecule or radical under these conditions. Therefore, within the lifetime of the excited dye or dye-oxy-radical, at least one of them has a greater chance of reacting with the trypsin.

at high trypsin concentrations. These observations argue against dye binding by the trypsin because if the dye is bound to the trypsin, this increased reactivity as a function of trypsin concentration would not be expected. Ghiron (12) has shown by dialysis techniques that with these particular dyes, strong dye-binding is most likely not involved in the photodynamic inactivation of trypsin. At present no satisfactory interpretation can be given of the data obtained in this study.

E. Interpretation of the Effect of pH on Trypsin Photoinactivation

Fig. 19 shows the quantum yield as a function of pH for the dyes used. Eosin Y- and MeB-sensitized systems show no measurable inactivation except at pH values of six or greater. FMN-sensitized systems show low but significant values of inactivation from pH 2 - 6. All three systems show high inactivation rates above pH 7.

The lack of inactivation in MeB- and eosin Y-sensitized systems under acid conditions may indicate that hydroxide ions are necessary in some way for the reaction to proceed with these dyes. It may also mean, however, that hydrogen ions are inhibitory rather than that hydroxide ions are needed. Since FMN-sensitized systems give small (but appreciable) quantum yields under acid conditions and high quantum yields above pH 7, it may be that FMN sensitizes by one mechanism on the acid side and another on the basic side of neutrality.

These data also may be interpreted in another way. Fig. 5 shows that the trypsin activity with respect to pH follows this same general form, i.e., low on the acid side and high on the basic side. Thus, trypsin

is only susceptible to photodynamic inactivation by MeB and eosin Y under pH conditions where it is enzymatically active. It may be that the trypsin molecule is folded in such a way under acid conditions that the active site is protected from photodynamic action. If the attack is not on the active site, then, it is hard to explain the pH effect. With FMN as sensitizer, it may be that photodynamic attack may occur to a limited extent at many different sites on the molecule, with the effect somehow being transmitted to the active site. Here again, as with MeB and eosin Y, the major point of attack under basic conditions could be the active site.

Over 200 dyes have been examined in this laboratory as sensitizers for the photodynamic inactivation of trypsin. Only two, riboflavin and FMN, show any activity under acid conditions. All of the rest behave like MeB and eosin Y. This is further evidence that more than one mechanism may be operating in photodynamic action, depending on the dye used. Also, only the flavin-sensitized systems show the damaged fraction phenomenon (12), i.e., the production of a class of trypsin molecules which, although active, are very labile to such agents as heat and urea. The other dyes examined do not produce the damaged fraction.

It is known that the kinds of free radicals produced by the interactions of ionizing radiation and water depend on pH, and this may be true in photodynamic action. The role of free radicals in photodynamic action is not understood in any useful way. However, pH can be envisioned as influencing the rate of photodynamic reactions in a variety of ways. First, many dye molecules are known to have different charges at different pH values, and second, the pK for a dye may be different for the excited state than for the ground state. Hence the dye and/or the trypsin, as mentioned earlier, may be affected by the pH.

F. Interpretation of the Effect of the Sodium Phosphate Buffer Concentration on Trypsin Photoinactivation

The quantum yield does not change when the sodium phosphate buffer concentration is varied from 0.00125 M - 0.125 M, when the pH is held constant throughout at 8.0. This is interpreted to mean that the phosphate ions themselves (H_2PO_4^- , HPO_4^{2-} and PO_4^{3-}) are not involved in the reaction but serve only in their buffer capacity to maintain the pH constant. Apparently also, they do not tend to inhibit by providing obstacles in the pathway of diffusion of the reactants.

G. Interpretation of the Effect of the Sodium Chloride Concentration on Trypsin Photoinactivation

Sodium chloride was varied from 0 - 1.0 M in the reaction solutions to see if these ions had any effect, since the phosphate buffers were used as sodium salts. At high concentrations the sodium chloride caused precipitation of MeB but not of eosin Y or FMN. There is no change in the quantum yield of the photodynamic inactivation of trypsin with respect to sodium chloride concentration except for that caused by the precipitation of MeB. This is taken to mean that sodium and chloride ions over the concentration range examined have no effect on the photodynamic process as such. Apparently, these ions cause no more hindrance to the diffusion of the reactants than did the phosphate ions.

H. Interpretation of the Effect of Temperature on Trypsin Photoinactivation

The quantum yield of the photodynamic inactivation of trypsin with all three dyes increases linearly with increasing temperature (Fig. 22). Arrhenius plots of the data give the following experimental activation energies, E_a : for the 150 μM FMN-sensitized system, 2.8 kcal/mole; for the 12.5 μM MeB-sensitized system, 4.8 kcal/mole; and for the 12.5 μM eosin Y-sensitized system, 3.7 kcal/mole. The magnitudes of these energies of activation are low for ordinary thermochemistry but much too high for photochemistry. Since these activation energies are low, in all probability no covalent bonds are broken as part of this thermochemistry, but rather, it involves some loosely bound states (hydrogen bonds, for instance). Alternatively, these low activation energies may represent simply increased diffusion or altered enzyme configuration due to higher temperature. Ghiron (12) reports much higher E_a values (11.7 kcal/mole) for FMN-sensitized trypsin inactivation systems at pH 4 - 5. The experiments in this thesis were carried out at pH 8.0. These differences in E_a due to pH changes may indicate that the trypsin configuration changes at the different pH values. Augenstine (114) found E_a values of 2.2 kcal/mole for the ultraviolet inactivation of trypsin at pH 4.5. An extensive study of temperature effects with respect to pH needs to be done.

I. Interpretation of the Effect of Sodium Iodide on Trypsin

Photoinactivation

If the triplet state is involved in photodynamic action, compounds that quench the triplet state would be expected to act as inhibitors in this system. Fig. 24 shows that sodium iodide inhibits trypsin photoinactivation using all three dyes as sensitizers. Sodium iodide causes MeB to precipitate out of solution analogously to sodium chloride. The figure also shows that the FMN-sensitized system is much more sensitive to sodium iodide than the other two systems; sodium iodide causes about seventy-five per cent inhibition at 0.0001 M ($100\text{ }\mu\text{M}$) in the FMN system, whereas 0.3 M sodium iodide is needed to cause about seventy-five per cent inhibition in the MeB and eosin Y systems. This would suggest, then, differential sensitivity to iodide ion in photodynamic systems due to different reaction mechanisms.

Iodide ion is thought to inhibit certain types of photochemical processes (trypsin photoinactivation in this case) because of its property of being a triplet quencher (29,63,98). Since iodide ion inhibits photodynamic action, this would indicate, then, that the excited long-lived state of the dye is the triplet state and that the iodide ion in some way alters the triplet state lifetime or chemistry such that trypsin inactivation is retarded because it makes possible non-radiative transitions to the ground state that could not occur otherwise. CMU (3-(p-chlorophenyl)-1,1-dimethylurea) has been shown to be inhibitory in some photochemical systems with flavins but not MeB (115) because it

quenches the flavin triplet state, again indicating differences in reaction mechanisms, as pointed out earlier.

J. Interpretation of the Effect of Histidine on Trypsin Photoinactivation

Fig. 25 shows the inhibitory effect of histidine on trypsin photodynamic inactivation. Histidine acts as an inhibitor presumably because it also is photooxidizable like trypsin (9-11). The mechanism, then, is supposedly one of competitive photooxidation. The degree of inhibition increases as the concentration of histidine increases in the reaction solution. In this work, significant degrees of inhibition were seen when the inhibitor was approximately twice as concentrated as the trypsin. The curves of quantum yield with respect to histidine concentration approach zero asymptotically, as would be expected in enzyme-substrate competitive inhibition. The kinetics for inhibition may be similar to those shown for enzyme-substrate inhibition (116,117), but in this study the dye is the catalyst and the trypsin and histidine are the substrate and inhibitor, respectively.

Other amino acids could probably function as competitive inhibitors of photodynamic action. Tyrosine, methionine, and tryptophan are photooxidizable (9-11) and hence would be expected to be inhibitory.

K. Interpretation of the Effect of AET on Trypsin Photoinactivation

Fig. 26 shows the inhibitory effect of AET on trypsin photoinactivation. AET causes the precipitation of eosin Y under our conditions. AET causes inhibition of trypsin photoinactivation in all three dye-sensitized systems.

AET is a classical radioprotective agent (94,95). It is shown in this study that AET also is protective in photodynamic systems. It presumably acts in a manner similar to histidine, i.e., by competitive inhibition, except for its causing the precipitation of eosin Y.

L. Interpretation of the Effect of Egg Albumin on Trypsin Photoinactivation

Fig. 27 shows the inhibitory effect of egg albumin on trypsin photoinactivation. Egg albumin causes no dye precipitation in any of the systems used herein. Egg albumin probably causes protection because it is photooxidized in a manner similar to trypsin and hence exerts competitive inhibition.

Histidine, AET, and egg albumin all act to protect trypsin from photoinactivation under these conditions. They all presumably act by competitive inhibition and presumably are photooxidized under these conditions, except for the precipitations, as noted earlier. This would predict, of course, that many other compounds could act similarly; among them, of course, would be aromatic amino acids, proteins, and various other compounds, all of which would be photooxidizable.

In general, compounds that are photooxidizable would be expected to act as inhibitors in this system unless other specific properties prevented it.

M. Possible Methods of Enhancement of Photodynamic Action

The foregoing discussions indicate that photodynamic action can be inhibited by triplet quenchers and by competitive inhibitors. The question then arises as to whether or not means can be found to increase rather than decrease the quantum yield of photodynamic action.

One method of enhancement would be to make the substrate more amenable to attack. This could perhaps be done by some type of chemical alteration or addition, depending on the nature of the substrate. In the case of proteins, conformational changes due to pH might also increase the photodynamic efficiency of the reactions.

Increased oxygen partial pressure also might prove helpful in some cases. Spealman and Blum (46) found that increased oxygen partial pressure increased the quantum yield of the photodynamic oxidation of the iodide ion to iodine. Ghiron (12) found the reverse for the riboflavin-sensitized photoinactivation of trypsin, from twenty-one to one-hundred per cent oxygen in the gas used; his data suggest an optimum oxygen concentration somewhere between these limits. Gaffron (118) found that the rate of the photodynamic excitation of horse serum increased with increasing oxygen partial pressure up to one atmosphere, suggesting that even higher oxygen partial pressure might increase photodynamic action in this case. Another method of enhancement of photodynamic action would be to increase the

intersystem crossing from the singlet excited state to the triplet excited state, since photodynamic action in most cases is thought to occur via the triplet state (see Fig. 1). This would also tend to reduce fluorescence (since it occurs from the singlet state) and increase phosphorescence (since it occurs from the triplet state) (29). McGlynn (119) has reviewed some facets of this problem. Various heavy atoms such as chlorine, bromine, and iodine substituted into the light-absorbing molecule in place of hydrogen or substituted into the solvent system enhance intersystem crossover to the triplet state. Light-excited complexes of polyacenes with such compounds as tetrachlorophthalic acid enhance intersystem crossing in the polyacenes with increased phosphorescence and decreased fluorescence. Weber (120) has shown that the fluorescence of DPNH varies greatly, depending on the solvent system. Spikes and Glad (15) have found that in the photodynamic inactivation of trypsin using xanthene dyes, the activity increases as the substituents on the xanthene increase. The parent compound fluorescein with no substitutions is a poor sensitizer. Eosin Y, containing four bromine atoms, is a very good sensitizer, for instance. Rose bengal is also very good (it contains four iodine and two chlorine atoms). Presumably, the substitutions enhance triplet population. A careful study of this series with respect to fluorescence and phosphorescence yields would solve this question.

Another method of enhancement of intersystem crossing can be accomplished by binding the sensitizer to some other compound. Bellin and Oster (37) have shown this enhancement of intersystem crossing in eosin bound to polyvinylpyrrolidone. Szent-Györgyi (121) and Theorell and Nygaard (122) have shown that in riboflavin-protein systems the fluorescence is quenched

over that for free riboflavin, presumably by increased population of the triplet state. Harbury et al. (61), have spectral and kinetic evidence to show complex formation between proteins and FMN. Whether enhanced intersystem crossing can occur in photodynamic action and cause increased photodynamic efficiency is not known.

N. Comparisons of Dye-Sensitized Trypsin Photoinactivation With Ultraviolet Trypsin Photoinactivation

Comparisons between dye-sensitized trypsin photoinactivation can be made with the more comprehensive studies of quantum yields of ultraviolet inactivation. The following section deals with this latter area in an attempt to correlate photodynamic inactivation with similar ultraviolet inactivation studies.

Quantum yield measurements have been made on many systems using ultraviolet light (123, 124). Values of 0.006 - 0.008 are representative for enzymes. Uber and McLaren (125) give the following as the quantum yield for the ultraviolet inactivation of trypsin: at 2537 Å, 0.017; at 2804 Å, 0.016, as measured by titration of the free acid groups produced from benzoylarginineamide by the enzyme. Verbrugge (126) gives values of 0.01 - 0.1 for the quantum yield for the ultraviolet inactivation of trypsin. He attributes the differences found from the work of Uber and McLaren as due to the differences in the absorption coefficients of the different trypsin preparations used. H. Goldenberg and A.D. McLaren (127) give 0.010 as the quantum yield for the ultraviolet inactivation of trypsin and 0.004 for chymotrypsin (106). McLaren (128) found that the inactivation of

trypsin using ultraviolet light was inhibited by the iodide ion. This is analogous to our work here using photodynamic action on trypsin. The quantum yield for the ultraviolet inactivation of trypsin is higher than that for the photodynamic inactivation of trypsin. This is reasonable because the dye intermediate is not necessary. The two systems are probably not too dissimilar with respect to the actual change in the trypsin molecule; peptide bond-splitting does not occur in either case.

Augenstine et al. (114,129), have studied the ultraviolet and ionizing radiation inactivation of trypsin. They find that certain sulfhydryl groups are formed from disulfide bonds and postulate their "weak link" hypothesis to explain their results, namely that energy is picked up in the molecule and transferred around until the weakest disulfide bonds are reached and then these are broken (sulfhydryl groups are formed). No sulfhydryl groups are formed during the photodynamic inactivation of trypsin.

It has been found that a general relationship exists between molecular weight and quantum yields for ultraviolet inactivation (123,124). Some examples for small proteins are: pancreatic trypsin inhibitor molecular weight = 6,000 and quantum yield = 0.03; insulin molecular weight = 12,000 and quantum yield = 0.045; ribonuclease molecular weight = 15,000 and quantum yield = 0.026; carboxypeptidase molecular weight = 31,600 and quantum yield = 0.001.

In general, as molecular weight increases, the quantum yield decreases. Log-log plots of molecular weight versus quantum yields give straight lines with negative slopes. Errera (130) found that photolysis of thymus DNA (molecular weight = 10^6) has a quantum yield equal to 10^{-6} at 2537 Å.

McLaren et al. (124,17), give quantum yields for ultraviolet inactivation of phages and viruses at about 10^{-4} - 10^{-5} . These nucleoprotein substituents may fit on the same curve. Setlow (131) has suggested that the quantum yields for ultraviolet inactivation of enzymes are roughly proportional to the cystine content and precisely proportional at 2537 Å. This same phenomenon can be seen to operate in photodynamic action. Small molecules such as amino acids have high quantum yields, i.e., 0.038 for tryptophan (52). Proteins are intermediate with values such as 0.006 for lysozyme (52) and trypsin as shown herein. When whole cells are considered such as by Blum (54), the quantum yields are very low, i.e., 1×10^{-10} .

This relationship may mean that as the size of the molecule or structure increases, the labile groups tend to be inside the molecule or structure and hence protected, or that the larger units are just intrinsically more stable, or that more damage has to occur before the change is noted. Many types and sizes of biological molecules need to be tried to see just how well this relationship holds.

0. Comments on the Mechanisms of Dye-Sensitized Trypsin Photoinactivation

All of the foregoing data and interpretations of trypsin photoinactivation help to understand this phenomenon, but they do not settle the question of the exact mechanism of action. This is inherent in any kinetic analysis. It is often stated that a kinetic analysis of a system will sometimes delineate what mechanisms are not possible, but will not be definitive in deciding just what mechanisms are operating.

The kinetics of dye-sensitized trypsin photoinactivation have been studied with respect to the four general mechanisms for photodynamic action outlined in the Introduction. It has been shown by Ghiron (12) that none of these mechanisms fits the data precisely. The work reported herein also corroborates this statement. Mechanisms I and II predicate a linear relationship between the rate of inactivation of trypsin and dye and enzyme concentration; Mechanism III postulates dye-binding, and Mechanism IV postulates the formation of hydrogen peroxide. The experimental results reported herein and by Ghiron (12) do not conform to any of these mechanisms. In all probability, we are not dealing with a single reaction but rather with a class of complicated reactions.

This question of exact mechanisms must be approached from other experimental points of view. For instance, Ghiron (12) approached the problem of dye binding using dialysis procedures and by measuring fluorescence and phosphorescence. The problem of the nature and the lifetimes of the excited dye species can be approached using flash photolysis (8,102). The nature of the substrate breakdown products can be approached biochemically, etc. All of these kinds of data, as well as classical kinetic studies, are necessary to describe adequately the exact reaction mechanisms.

VI. SUMMARY

The quantum yield of the photodynamic inactivation of trypsin has been studied using MeB, eosin Y, and FMN as sensitizers. The reaction was studied for each dye with respect to several kinetic variables: stirring rate, dye concentration, trypsin concentration, light intensity, pH, sodium phosphate buffer concentration, sodium chloride concentration (ionic strength), temperature, and various protective agents including sodium iodide, histidine, AET, and egg albumin. The reaction in all cases was carried out in air, and molecular oxygen is used up in the process.

In stirred systems the time-course of the photodynamic inactivation of trypsin was first-order within experimental error under all conditions examined. In unstirred systems the results with MeB and eosin Y were the same as in stirred systems. With FMN as sensitizer, however, the rate of inactivation in unstirred systems was very low and the reaction deviated markedly from a first-order process. On the basis of other studies in this laboratory, these latter results could be interpreted as demonstrating a different dependence on oxygen partial pressure for the FMN-sensitized system.

The effect of dye concentration on the quantum yield of trypsin photoinactivation was studied. MeB-sensitized systems have a quantum yield maximum of 0.0014 at 12.5 μM , eosin Y-sensitized systems have a quantum yield maximum of 0.0027 at 25 μM , and FMN-sensitized systems have a maximum value of 0.0023 at 150 μM . These data indicate that the three mechanisms of action are probably different.

The quantum yield is independent of light energy absorbed over the range studied ($0 - 0.01 \text{ watts/cm}^2$) for all three dye-sensitized systems.

The quantum yield decreases with increasing trypsin concentration over the range of $5 - 42 \text{ }\mu\text{M}$ trypsin for all three dye-sensitized systems. These results are difficult to interpret at this time.

The quantum yield of trypsin photoinactivation was examined over the pH range $2 - 10$. No inactivation is evident for MeB- or eosin Y-sensitized systems at pH values below about 6. FMN-sensitized systems show low but significant quantum yield values from pH $2 - 6$. All three dye-sensitized systems show high quantum yield values above pH 7. FMN may operate by different mechanisms on the acid side and basic side of the pH scale. These results can be interpreted to mean that the enzyme is folded in such a way that it cannot be inactivated readily in acid.

The quantum yield in all three dye-sensitized systems has been shown to be unaffected by changes in the concentration of sodium phosphate buffer present over the range $0.00125 \text{ M} - 0.125 \text{ M}$ when the pH is held constant. Similarly, sodium chloride concentration up to 1.0 M has no effect on the eosin Y and FMN systems. MeB is precipitated out at high salt concentrations.

The quantum yield of trypsin photoinactivation is an increasing linear function of temperature for all three dye-sensitized systems. Temperature studies give experimental activation energies, E_a , of from $2 - 5 \text{ kcal/mole}$ for the photodynamic inactivation of trypsin. These values indicate either rate-limiting thermochemistry of some sort or the involvement of diffusion-controlled processes that require little energy.

Many compounds are known to inhibit photodynamic action; these act by various mechanisms. Sodium iodide was found to inhibit trypsin photoinactivation in FMN- and eosin Y-sensitized systems, presumably by quenching the long-lived excited (triplet) state of the dye; it causes precipitation of MeB in a manner analogous to sodium chloride. The MeB- and eosin Y-sensitized systems are inhibited by molar amounts of sodium iodide, while the FMN system is inhibited strongly by only micromolar amounts. These data also show the differences in the mechanisms between the FMN-sensitized system and the other two systems.

Histidine, S-(2-aminoethyl)isothiuronium bromide hydrobromide (AET), and egg albumin were used as inhibitors; they presumably act as inhibitors because they can be competitively photooxidized. Histidine and egg albumin cause inhibition in all three dye-sensitized systems. AET inhibits MeB- and FMN-sensitized systems; it causes precipitation of eosin Y.

All of these data are useful in evaluating possible reaction schemes for photodynamic action because any mechanism proposed must fit these kinetic parameter limitations. No currently proposed mechanism fits our experimental observations exactly, and further, these data support the conclusion that the mechanisms of action are different, depending on the dye used as the sensitizer. Additional types of studies will be needed to determine the exact reaction mechanisms.

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