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The Effect of Ultraviolet Irradiation on the Hydrolytic Activity of Trypsin. Protection by S, 2-Aminoethylisothiouronium Bromide Hydrobromide

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THE EFFECT OF ULTRAVIOLET IRRADIATION ON THE HYDROLYTIC ACTIVITY OF TRYPSIN. PROTECTION BY S, 2-AMINOETHYLISOTHIOURONIUM BROMIDE HYDROBROMIDE

by

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LIFE

Albert J. Iammartino was born in Tampa, Florida on December 9, 1944. In June, 1962, he graduated from St. Peter's High School, Staten Island, New York. From 1962 to 1966 he attended Wagner College, Staten Island, New York, and received the degree of Bachelor of Science.

In September, 1966, the author entered the Department of Biochemistry and Biophysics at the Loyola University Stritch School of Medicine. From 1966 to the present, he has been a National Institute of Health Trainee in Biochemistry.

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. ABBREVIATIONS

AET	S, 2-aminoethylisothiouronium bromide hydrobromide
APT	S, 3-aminopropylisothiouronium bromide hydrobromide
GED	bis(2-guanidoethyl) disulfide
MBA	4-mercaptobutylamine
MEA	2-mercaptoethylamine
MEG	2-mercaptoethylguanidine
MPA	3-mercaptopropylamine
TAME	p-toluenesulfonyl-L-arginine methyl ester

CHAPTER I

INTRODUCTION

Ionizing radiation in the course of dissipating energy produces alterations in irradiated tissues. In no cases, however, have the details of the underlying interactions or the electronic processes been worked out. The initial chemical changes may result in cellular damage and death. In mammalian systems, the end effect might very well be the death of the organism. The discovery that systems in vivo and in vitro can be partially protected against the deleterious effects of ionizing radiation by prior administration of certain, specific chemical compounds is therefore of great interest.

The Protective Compounds

As early as 1942, Dale in England demonstrated that the addition of colloidal sulfur or thiourea to an aqueous solution of carboxypeptidase or d-aminoacid oxidase decreased the x-ray induced inactivation of these substrates (1). Cyanide has been shown to be another excellent radio-protector in living systems (2, 3). Ever since the discovery that cysteine and glutathione could reduce acute radiation death in mammals, the sulfhydryl containing compounds have been exhaustively investigated and have been found to be the most effective (4). Spurred on by this information, Bacq and associates examined a large number of sulfhydryl compounds and amines for protective activity. Their investigations revealed that 2-mercaptoethylamine (MEA) was a more powerful protector than all those

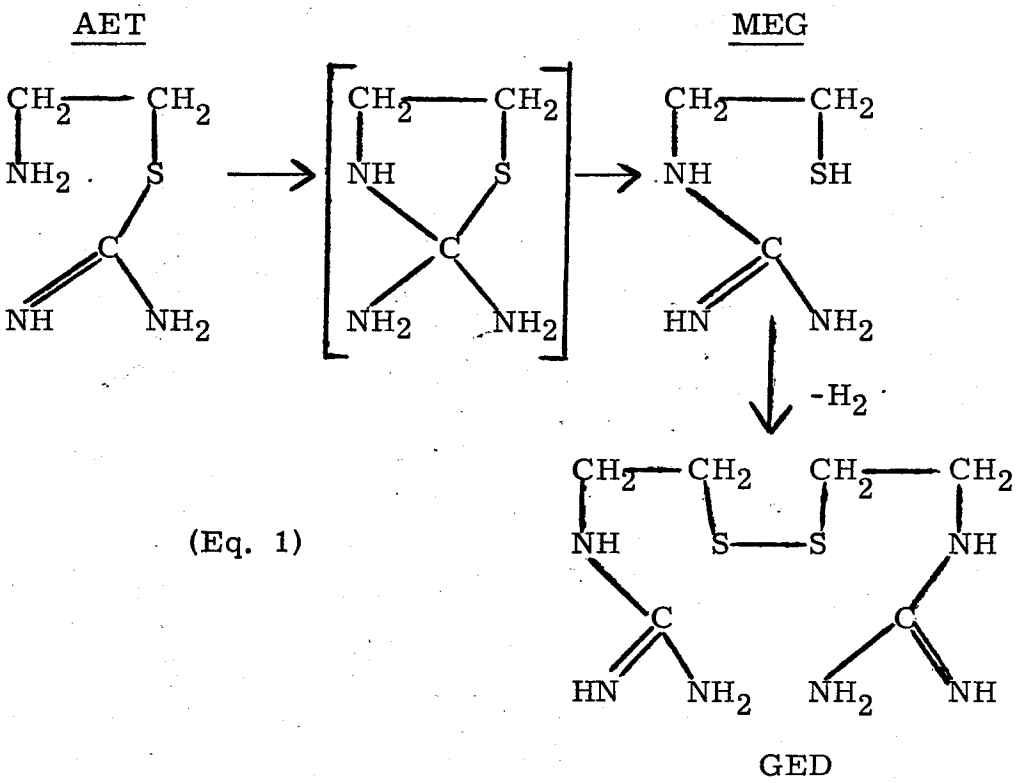
previously tested (5). Extended survival was observed in lethally irradiated mice given the compound immediately before irradiation. In contrast to cystine, the disulfide corresponding to MEA, cystamine, was equally effective.

By variation in chemical structure of MEA, Doherty, Burnett, and Shapira attempted to define the necessary parameters for protective activity (6). The study revealed the necessity for a basic amino group for maximum activity. Acylation of the amino group yielded compounds with markedly decreased activity. The addition of a carboxyl group to yield cysteine also reduces the basic character of the amino group and yields a less protective compound. On the other hand, a partial restoration of the basicity by conversion of the carboxyl to the thiyl ester increases the protective ability. 3-Mercaptopropylamine (MPA) in which the functional groups are separated by a three carbon chain a more efficient protector than MEA by a factor of two. Further separation of the amino and thiol functions as in 4-mercaptobutylamine (MBA) reduced the protective activity to one-fifth that of MEA. It thus appeared that both the sulfhydryl and the amino groups are necessary for protection. This is in agreement with the observations of Alexander et. al. (7). In this class of compounds, the combination of a basic and a sulfhydryl group separated by not more than three carbon atoms appears to offer the best radioprotective properties.

In the course of structural studies on the mercaptoalkylamines, it was discovered that certain isothiouronium compounds exibited an even greater effectiveness as radioprotectors. Doherty and Burnett presented evidence that two such compounds, S, 2-aminoethylisothiouronium bromide hydrobromide (AET) and S, 3-aminopropylisothiouronium bromide hydrobromide (APT) were more effective than MEA (8). AET and APT were later examined more closely to determine the active form of these compounds in solution. A larger series of isothiouronium compounds were also prepared in an attempt to correlate structure with protective activity (9).

Since in addition to the isothiuronium group a basic group is necessary for protection (8) a series of isothiouronium salts was prepared with the amino group of AET being replaced by nitrile, hydroxy, and carboxyl groups. None of these modifications yielded compounds with any marked protective activity. The N-acetyl derivative of AET like the corresponding acyl compound, was also ineffective. To test the requirement for the amino group, the hydrogen atoms on the amino group were replaced by alkyl, or substituted alkyl groups. The dialkyl, substituted alkyl, and trialkyl, were ineffective. The replacement of the hydrogens by cyclic nitrogen compounds also resulted in the synthesis of an ineffective compound.

Chemical evidence indicated that AET had undergone intratransguanylation at neutral pH's to form 2-mercaptoethylguanidine (MEG). It thus appears that the protective compound is in fact MEG obtained from in situ rearrangement of AET. A thiol disulfide equilibrium shown in equation 1 was demonstrated for both AET and APT (10):



(Eq. 1)

The disulfide form of MEG, bis(2-guanidoethyl) disulfide has been reported (9) as offering slightly less protection than MEG. Other reports however indicate that both are equally effective when given intraperitoneally while GED is less active orally (11). This difference might be due to metabolic and/or absorption differences.

A variety of substituted aminoalkylisothioureas related to AET and APT have also been studied for protective activity and toxicity. When toxicity is taken into account, the most effective compound appears to be MEG. The superiority of MEG as a protective agent is attributed to the structural characteristic of this compound which allow the guanido and thiol groups to come into close proximity. The proximity of the electrophilic guanido group increases ionization of the thiol group which may promote disulfide interchange between the protective thiol and the protein disulfide bonds (12). This interchange which is discussed in the following section may be important for the mechanism of protection of disulfide containing proteins.

The Mechanism of Action of the Protective Agents

The collection of experimental data on the molecular events occurring in cells during the process of irradiation is clearly an extremely difficult task. Most postulates concerning the mechanism of protection rest on indirect evidence from model experiments in vitro. Several

contributing mechanisms have been postulated to explain the protective effect of a specific compound in vivo. It may be assumed that different mechanisms predominate for the different types of protective agents.

For irradiations in solution the protective agent may moderate irradiation damage by combining with free radicals formed by the radiolysis of water (13). In recent years, it has been demonstrated that the direct interaction of the ionizing radiation with the target material or cell constituents and damage that might be mediated by hydroxyl and hydroperoxyl radicals produced from radiation of water are equally important (14).

Shapiro and Dickens have shown that both MEG and GED are capable of reacting with the oxidizing free radicals formed in irradiated solutions in vitro (15), but it is an open question whether this also occurs in vivo where the local concentration of protective agents in the tissue is exceedingly low and the reactivity towards the radicals is also low. However, since organic compounds will compete with a target molecule for free radicals, the radical scavenger mechanism must, to some degree, be operative in cells and tissues.

Removal of oxygen may also contribute to the protection. The fact that most radiation damage in vivo and in vitro is reduced when the irradiation is carried out under reduced oxygen pressure has been central to the idea that protection occurs via a lowering of the cellular oxygen

tension. Since the effects of oxygen deprivation and presence of thiol are additive in mice, more than a single mechanism of protection might be operative (16). Some in vitro experiments have been interpreted as indicative that protection is the result of progressive lowering of the oxygen tension caused by the spontaneous in situ oxidation of the thiol (17).

Another factor accounting for the protective effect of thiols may be the chemical modification of target molecules by the protective agent. Sulfhydryl and disulfide containing agents have been shown to attach to tissue constituents through the in vivo formation of mixed disulfide bonds (18). A mechanism of protections based on mixed disulfide formation has been postulated on the basis of data obtained with cysteamine and cysteine (19). During the period of protection, the protective compounds of the cysteine-cysteamine group exist in the body largely in the form of mixed disulfides with the disulfide and sulfhydryl groups of tissue protein. This binding is temporary and is reversed by disulfide-reducing enzymes in the tissue.

When a mixed disulfide interacts with a free radical originating from a water molecule one of the sulfur atoms is oxidized to a sulfinic or sulfonic acid group and the other is reduced to a thiol. Statistically, this would promote the regeneration of the native protein sulfhydryl group during one half of the incidents of interaction. The protein sulfur atom

would be irreversibly oxidized during the other half of the incidents. Disulfide interchange may also provide protection to proteins against the direct effect of ionizing radiation in that the energy absorbed by the protein molecule could be transferred to the mixed disulfide bond and be dissipated during the rupture of the disulfide bond without damaging the molecule.

In support of the mixed disulfide mechanism, it has been shown that cysteamine becomes bound to the proteins of the serum, erythrocytes, and cellular constituents by disulfide bonds (20). In fact the active protective compounds in vitro form mixed disulfides with cystine and oxidized glutathione while the non-protective do not (21). Correlations between in vivo protection and the in vitro rate of mixed disulfide formation can be made (20).

However, some doubt exists as to the significance of this evidence. First, the time correlation between protection and protein binding does not appear particularly convincing. In addition, several compounds such as cystine which form mixed disulfides do not exhibit protective properties while many synthetic polymers, dry or in solution, in vivo and in vitro are well protected by MEA, cysteine, and MEG. These polymers contain no sulfur and cannot possibly form mixed disulfides. Finally, although some investigators insist that biological target molecules belong to the catalytic system of the cell, (13) indications are that the enzymic or co-enzymic

activities of the cell are not decreased, but in fact may be increased at the time of irradiation (18). It must also be pointed out that in general blocking thiol groups does not necessarily result in enzymes with increased resistance against irradiation induced deactivation. On the basis of this evidence, little can be said with certainty regarding the mechanism of protection except that disulfide formation may be the first step in radioprotection in mammals.

CHAPTER II

MATERIALS AND METHODS

General Experimental Procedure

Rate studies are employed to relate the degree of photochemical damage in the protein as indicated by loss of the hydrolytic activity of trypsin in the absence of MEG to the degree of protection in the presence of this thiol. To accomplish this, a series of dilute trypsin solutions in phosphate buffer, pH 4.0 are prepared. A precursor of the thiol, AET, is dissolved in phosphate buffer (pH 6 to 7). At this pH a quantitative rearrangement to MEG takes place. The experimental samples, consisting of trypsin and trypsin solutions containing thiol are degassed and irradiated on a turntable for varying periods of time. Control solutions consisting of trypsin and trypsin containing thiol are treated exactly as the experimental samples but are not irradiated. The enzymatic activity of each sample is measured before and after irradiation by determining the rate of hydrolysis of p-toluenesulfonyl-L-arginine methyl ester (TAME).

Assuming that the thiol protects via rapid hydrogen transfer reactions, the protected enzyme should serve as an acceptor of thiol hydrogen. In order to test this assumption, the photoreaction is carried out in the presence of tritium oxide which is expected to allow a rapid exchange of tritium with thiol hydrogen. Irradiations are subsequently

carried out as previously described and part of each sample is analyzed for enzymatic activity. Excess tritium oxide and exchangeable tritium are separated by column chromatography and equilibrium dialysis. Concentrations of protein-bound non-exchangeable tritium are then determined by liquid scintillation counting. Samples irradiated in the presence and absence of MEG, as well as trypsin blanks containing MEG but not subjected to irradiation, are counted.

Preparation of MEG Stock Solution

AET (0.014 g., Sigma Chemical Company, St. Louis, Mo.) was dissolved in 5 ml of 0.066 M sodium phosphate (pH 10.5). The concentration of MEG obtained in the stock solution was $1x10^{-2}$ M. The resulting solution (pH 6.9) was immediately adjusted to pH 6.3-6.5 and was allowed to stand at room temperature for one hour to assure complete conversion of AET to MEG. The oxidation of MEG to GED which readily occurs in more alkaline solutions is apparently not appreciable at this pH. It has been reported, however, that small amounts of GED (up to 10%) may be present under these conditions (11).

Preparation of Trypsin Solutions

Trypsin (0.040 g., twice crystallized and salt-free, Worthington Biochemical Corporation, Freehold, N. J.) was dissolved in 10 ml of phosphate buffer (0.066 M) at the pH selected for each experiment. The

mixture was allowed to stand at room temperature until all materials were completely in solution. The concentration of the trypsin solution was 16.8×10^{-5} M. The solution normally was used within one hour from the time of preparation.

Preparation of the Experimental Solutions

Solutions to be irradiated and blanks were prepared by dilution of the 16.8×10^{-5} M trypsin solution. A 5 ml aliquot of trypsin was pipetted into a 10 ml volumetric flask. The pH was adjusted with H_3PO_4 (first with 1N and subsequently with 0.1N acid). The volume was completed with NaOH (1N, 0.1N, and 0.01N base in sequence). The concentration of this solution was 8.4×10^{-5} M (0.2% of trypsin expressed as weight to volume ratio).

For the trypsin solution containing thiol, a 1.0 ml aliquot of the concentrated MEG is added to the original 16.8×10^{-5} M trypsin solution. The final concentrations of trypsin and MEG, after volume and pH adjustment are 8.4×10^{-5} M and 1.0×10^{-3} M respectively.

Assay for Enzymatic Activity

Enzymatic activity is assayed by measurement of the rate of hydrolysis of a suitable substrate. The chosen method of assay is based on the change that occurs in the ultraviolet absorption during the hydrolysis of an amino acid ester, p-toluenesulfonyl-L-arginine methyl ester (TAME)

(22). The change in the difference between the absorbance of the ester and that of the corresponding acid during hydrolysis of the ester is monitored at 247 mμ at which wavelength this difference is maximum.

The substrate, TAME·HCl (0.0379 g., Sigma Chemical Company, St. Louis, Mo.) is dissolved in 10 ml of distilled water to give a 0.01 M solution. The trypsin solution to be tested (8.4×10^{-5} M) is diluted by pipetting 0.1 ml of the sample into a 25 ml volumetric flask and completing the volume with 0.001 M HCl. Thus, the concentration of the assay solution is 8×10^{-6} g./ml. The assay is carried out by a Cary 15 recording spectrophotometer. A blank is prepared by thoroughly and rapidly mixing 0.3 ml of substrate solution, 0.1 ml HCl (1×10^{-3} N) and 2.6 ml of buffer (0.04 M Tris, 0.01 M $CaCl_2$, pH 8.1) and placed in the reference compartment of the spectrophotometer. The assayed sample is similarly prepared by mixing 0.3 ml of substrate in 2.6 ml of buffer with 0.1 ml of the enzyme solution and placed in the sample compartment. The reaction is allowed to continue for approximately ten minutes while the absorbance at 247 mμ is being recorded. At the initial stage of hydrolysis a linear plot of absorbance versus time is obtained. Rates of hydrolysis are calculated on the basis of absorbance change per minute per microgram of enzyme.

Preparation of Solutions for Irradiation

Irradiations are carried out in custom-made quartz tubes connected to a Teflon-stoppered glass vacuum-holding valve (Lab Crest, Quick Opening Threaded Glass Valve, Fisher and Porter, Warminster, Penna.) via a vycor-glass graded seal. The sample holding portion of the tube is one inch in length and a quarter inch in diameter. Magnetic stirring bars are placed at the bottom of the tube and a 1 ml sample is introduced by a long tip seriological pipette. The tubes are attached to a manifold connected to a vacuum pump, placed in an ice-methanol bath at -15° C., and the samples are allowed to slowly freeze. Once the samples have been frozen, the tuves are degassed. The valves of the tubes are subsequently closed and the solutions are allowed to melt. The freeze, degass, and melt, sequence is usually repeated three times.

Irradiation Apparatus

The apparatus consists of a sample holder and an ultraviolet source. The source is a high intensity quartz lamp rated at 120 microwatts/cm^2 at a distance of 18 cm at 254 mu (Ultraviolet Products Incorporated, San Gabriel, Calif.). The sample holder is a custom-made rotating aluminum disk with holes provided about its periphery through which irradiation tubes can be fitted. Two lamps are positioned in the center of the turntable. The distance between the samples and the lamp

10 mm. Samples are stirred by two stationary magnetic stirrers placed underneath the tubes. Irradiations are carried out in a cold room. The ambient temperature is maintained at 5°C. by a properly placed air blower. Later irradiations were carried out in a Rayonet photochemical reactor (Model MGR-100, Southern New England Ultraviolet Company, Middletown, Conn.). This apparatus insures equal radiation of all samples as they rotate past the ultraviolet source. Five of the reactor's sixteen available lamps were used for irradiations.

Assay of Irradiated Samples

Each sample is assayed prior to, and following, the irradiation procedure. Depending on the extent of deactivation caused by the irradiation the amount of enzyme solution used for the assay must be properly adjusted so that a conveniently measurable rate can be obtained. Amounts used in these assays varied from 0.1 to 0.5 ml corresponding to dilution of the trypsin (3.36×10^{-7} M) solutions between six and thirty times.

Irradiation of Trypsin Solutions

A series of irradiations were carried out under varying conditions of pH and for various lengths of time to determine the conditions for maximum protection of trypsin by MEG. For irradiations at pH 4.0, 5.0, and 5.5, a stock solution was prepared by dissolving trypsin (0.040 g.) in buffer (0.066 M NaH_2PO_4, pH 4.0). At pH 6.0, the stock solution was

prepared in 0.066 M disodium phosphate, (pH 7.5).

Irradiation of Trypsin in the Presence of Tritium Oxide

To a trypsin solution (1.0 ml, 4.2×10^{-4} M) and to a corresponding sample of equal concentration and volume of trypsin with added MEG (1×10^{-3} M), at pH 4.0, tritium oxide (500 mc, Schwarz Bioresearch, Milwaukee, Wis.) was added. The enzymatic activity of each sample was obtained. The samples were degassed, placed in a fixed position in front of the lamp, and irradiated for a period of three hours. The control solution, containing identical concentrations of trypsin and MEG was handled in the same fashion but was not irradiated. Aliquots from the three samples were assayed for hydrolytic activity and the remainder of each sample was used to determine the extent of incorporation of tritium into the enzyme.

The irradiated samples and a control sample containing trypsin and MEG but not subjected to irradiation were individually diluted to a volume of 2 to 3 ml, placed inside equilibrated (in H_2O) cellulose dialysis tubes and dialyzed against buffer (NaH_2PO_4, 0.066 M, pH 2.0). Twelve buffer changes (4 liters each) were performed over a period of 72 hours. The samples were subsequently dialyzed versus a dilute solution of HCl (4 liters, pH 2.0). The dialysate was changed sixteen times. All samples were monitored for tritium loss by counting the dialysate before each change. After the final dialysis, aliquots from each sample were counted.

Other aliquots were placed on a chromatography column packed with Sephadex G-25 (coarse grade). The column was eluted with a sodium phosphate solution, (0.066 M, pH 4.0), at a flow rate of 1.2 ml/minute. The eluant was monitored spectrophotometrically at 225 mμ.

The collected trypsin samples were further dialyzed (at elevated temperatures "70-80°C.") versus a dilute solution of HCl (200 ml, $1x10^{-2}$ M, pH 2.0). The control solution (18 ml) was dialyzed for eight hours at 80°C. with ten changes of dialysate. The irradiated trypsin and the thiol containing trypsin samples (18 ml each) were dialyzed for four hours at 70°C. with four changes of solution.

Dialysis in the Presence of Urea

Urea (8.0 g., Mann Laboratories, New York, N. Y.) was dissolved in each of the irradiated samples which were previously dialyzed at elevated temperatures. The solutions were again dialyzed against 8 M urea for a total of sixteen hours. During dialysis, the dialysate (100 ml) was replaced twice with fresh urea solution. The dialysis was continued against distilled water for a period of twenty-two hours. The dialysate (100 ml) was replaced five times over this period. The concentration of trypsin was calculated from the absorption spectrum of a 1.0 ml aliquot removed from each sample. The sample was then placed in 15 ml of liquid scintillation fluor (dioxane, napthalene, and 2, 5-Diphenyloxazole) and the radioactivity was assayed on a Beckman LSC-50

Liquid Scintillation Spectrometer.

In later experiments, 0.5 ml aliquots of the previously dialyzed trypsin (4.2×10^{-4} M) and trypsin containing thiol samples were diluted to 18 ml with urea. These samples and the corresponding blank were dialyzed versus 8 M urea for sixteen hours. The urea solution was changed twice and dialysis was continued versus distilled water for an additional five hours. Water was replaced four times. After the final dialysis, the exact concentration of each sample was calculated spectrophotometrically. Radioactivity determinations were carried out as previously.

CHAPTER III

EXPERIMENTAL RESULTS

The Inactivation of Trypsin by Ultraviolet Light

Initial irradiation experiments were carried out at neutral pH on a custom made irradiation turntable. Irradiation of trypsin for a period of three hours led to partial deactivation of the enzyme. Trypsin at a concentration of 8.4×10^{-5} M retained 45% of hydrolytic activity present before irradiation while an identical trypsin sample containing 1×10^{-3} M MEG retained 53% of the original activity. These irradiations and all subsequent work was carried out with degassed samples in order to avoid air oxidation of the protective thiol.

Gel filtration elution patterns indicated that in addition to a main peak attributed to trypsin, several smaller molecular weight fractions are present in the irradiated samples indicative of partial hydrolysis of the enzyme. Previous in vivo studies with trypsin have been carried out by necessity at physiological pH (6). In vitro studies at pH 7.0 have also been reported (23). At neutral pH trypsin is known to catalyze its own hydrolysis. Self-digestion however, does not appear to introduce serious complications for samples exposed to irradiation for brief periods of time.

Dependance of Trypsin Inactivation on pH

The effect of hydrogen ion concentration on the deactivation and protection of trypsin by MEG was determined by performing irradiations at varying pH conditions. The concentrations of trypsin and thiol were

8.4×10^{-5} M and 1×10^{-3} M respectively, and samples were irradiated for a period of five hours. The results are summarized in Table I. The degree of irradiation induced damage is indicated by the rate of the trypsin catalyzed hydrolysis of p-toluenesulfonyl-L-arginine methyl ester (22). The hydrolytic activity is calculated from the change in absorbance per minute per microgram of enzyme. Percentage of remaining activity is defined as the ratio of activity of the irradiated sample over the activity of the unirradiated control multiplied by one-hundred. Percentage deactivation is defined as per cent of remaining activity subtracted from one-hundred.

Examination of Table I reveals that the rate of trypsin deactivation increases with increasing pH. The same observation can be made about trypsin irradiated in the presence of MEG. However, the percentage of remaining activity is consistently higher for the trypsin sample containing thiol regardless of pH. Per cent protection is calculated by dividing the difference between hydrolytic activities of trypsin irradiated in the presence and absence of thiol by the activity of irradiated trypsin. The per cent protection versus pH is plotted in Figure I.

In order to eliminate possible complications resulting from self-digestion of trypsin at neutral pH's, further studies were carried out at pH 4.0. As the results in Table I indicate, at this pH, conditions for maximal protection are also maintained.

TABLE I

PROTECTION OF TRYPSIN (8.4×10^{-5} M) FROM ULTRAVIOLET INDUCED DEACTIVATION IN THE PRESENCE OF MEG (1×10^{-3} M) AT VARYING pH CONDITIONS

pH	% ACTIVITY REMAINING		% PROTECTION
	TRYPSIN	TRYPSIN-MEG	
4.0	10.0	15.4	54
5.0	8.1	9.1	13
5.5	6.9	9.6	39
6.0	6.8	8.2	21

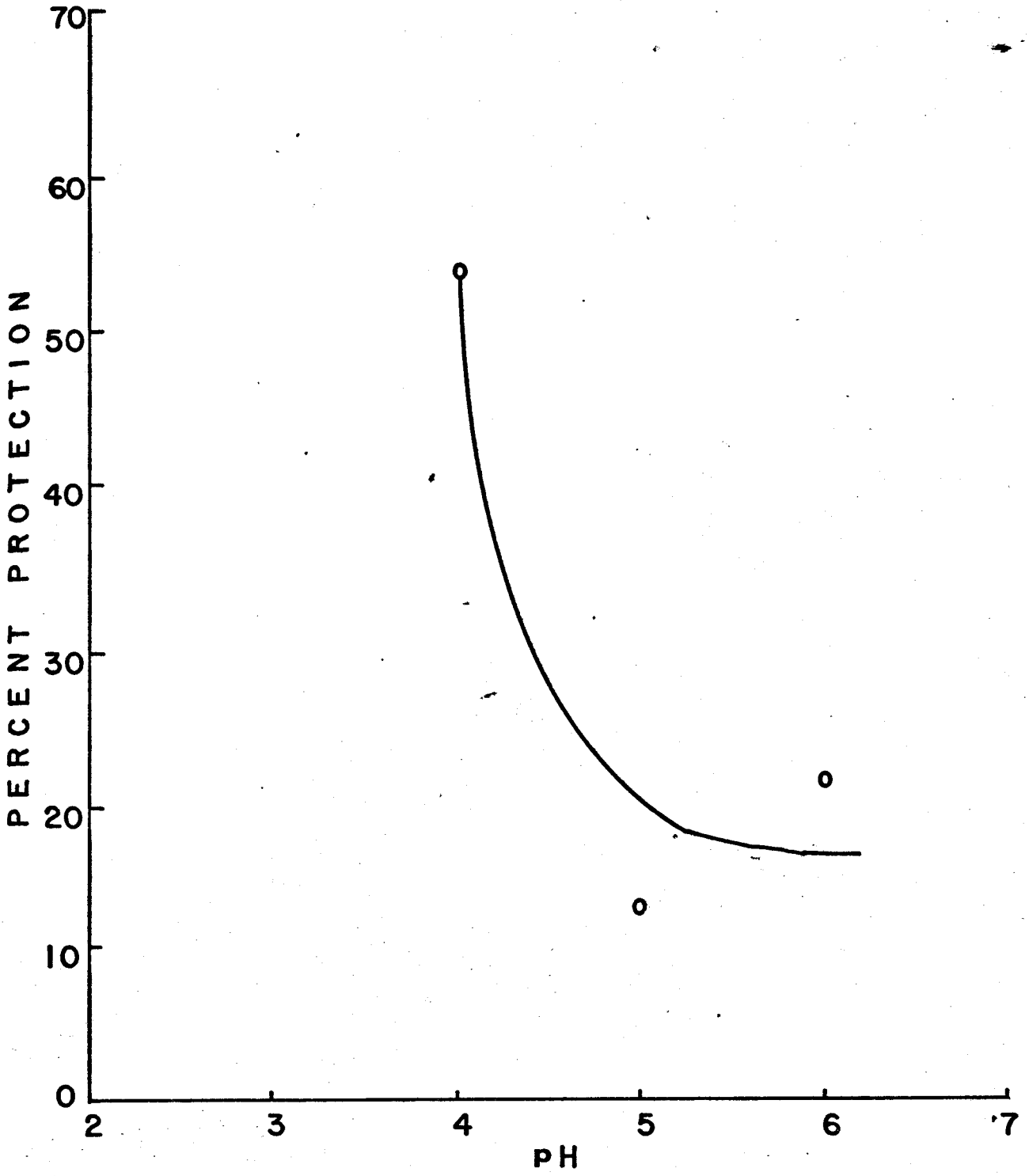


FIGURE 1: PROTECTION OF TRYPSIN (8.4×10^{-5}M) FROM ULTRAVIOLE INDUCED DEACTIVATION IN THE PRESENCE OF MEG (1×10^{-3}M) AT VARYING pH CONDITIONS

Irradiations of trypsin solutions (4.2×10^{-4} M) and trypsin solutions containing 1×10^{-3} M MEG were carried out in a higher energy output Rayonet irradiation chamber at pH 4.0 for varying periods of time. The experimental conditions were identical for all samples. The results are shown in Table II. The original activity of trypsin was reduced by approximately 30% after irradiation for twenty minutes. No appreciable protection was afforded by MEG in a trypsin sample irradiated for the same period of time. After thirty and forty minutes of irradiation approximately 50% of the original activity of the trypsin remains. Identical samples containing thiol maintain, respectively, 7% and 16% higher hydrolytic activities. Protection is not apparent for a sample irradiated for fifty minutes while both samples irradiated for seventy and eighty minutes are markedly protected by thiol. The apparent inconsistancies in the rates of deactivation of samples irradiated in the presence of thiol may be the result of an artifact introduced by the presence of varying amounts of residual atmospheric oxygen in the irradiated solutions. Although the effect of oxygen was not studied in the present system, it is reasonable to assume that it may lead to the oxidation of the added thiol during irradiation. Since the resulting oxidation products are not expected to be effective protectors, the presence of small amounts of oxygen may result in an overall decrease in protection. This effect has previously been observed during the mesitylthiol inhibition of the photoreduction of benzo-

TABLE II

IRRADIATION INDUCED DEACTIVATION OF TRYPSIN (4.2×10^{-4} M) IN THE PRESENCE AND ABSENCE OF MEG (1×10^{-3} M)

IRRADIATION TIME (min.)		% ACTIVITY REMAINING	% DEACTIVATION	% PROTECTION
20	TRYPSIN	65	35	
	TRYPSIN-MEG	65	35	NONE
30	TRYPSIN	54	46	
	TRYPSIN-MEG	58	42	7
40	TRYPSIN	50	50	
	TRYPSIN-MEG	58	42	16
50	TRYPSIN	38	62	
	TRYPSIN-MEG	38	62	NONE
70	TRYPSIN	31	69	
	TRYPSIN-MEG	37	63	19
80	TRYPSIN	29	71	
	TRYPSIN-MEG	35	65	21

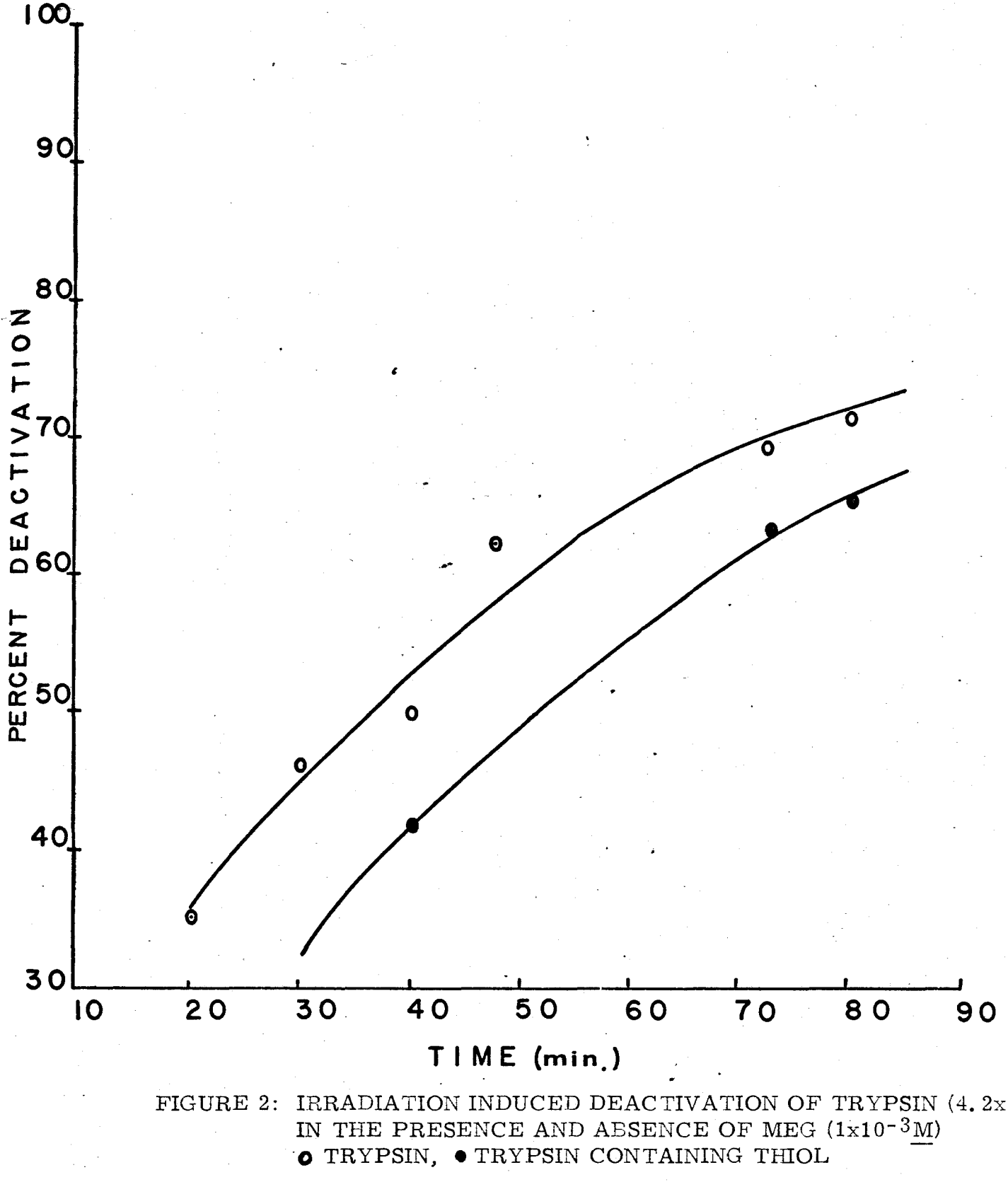


FIGURE 2: IRRADIATION INDUCED DEACTIVATION OF TRYPSIN (4.2x1
IN THE PRESENCE AND ABSENCE OF MEG ($1x10^{-3}$M)
o TRYPSIN, • TRYPSIN CONTAINING THIOL

phenone in methyl-2-octyl ether (24).

Tritium Incorporation Studies

Irradiation of a trypsin solution (1.0 ml, 4.2×10^{-4} M) for a period of three hours resulted in extensive loss of enzymatic activity. Under these conditions 2.5% of the original activity was retained while a trypsin solution containing MEG (1×10^{-3} M) retained 4.9% of the original activity. Tritium oxide (500 mc) was added to each of the above samples and also to a trypsin-thiol solution of the same concentration which was not exposed to irradiation. Prior to dialysis, each of the samples was diluted by a factor of 2.5. After initial dialysis of the trypsin solutions 0.1 ml aliquots of the irradiated trypsin solutions and the trypsin blank were analyzed for residual radioactivity. The results are summarized in Table III.

Further separation of residual tritium oxide and exchangeable tritium was effected by fractionations of 0.5 ml aliquots on a chromatography column packed with Sephadex. The elution of the column was monitored spectrophotometrically at 225 mμ and fractions corresponding to trypsin absorption were collected (20 ml) and combined. Radioactivity counting was performed on 0.1 aliquots of each sample. The observed readings are included in Table III and indicate that the chromatographic procedure led to further removal of free tritium oxide and exchangeable tritium from the trypsin. To assure complete removal of exchangeable tritium, additional dialyses were carried out in 8M urea solution. This protein

TABLE III

INCORPORATION OF TRITIUM INTO IRRADIATED TRYPSIN (4.2×10^{-4} M) AND TRYPSIN IRRADIATED IN THE PRESENCE OF MEG (1×10^{-3} M)

METHOD OF SEPARATION	SAMPLE	COUNTS/MIN/MG TRYPSIN
Dialysis	Irradiated Trypsin	1,048,000
	Irradiated Trypsin-MEG	863,200
	Unirradiated Trypsin-MEG	394,000
Dialysis and Chromatography	Irradiated Trypsin	560,000
	Irradiated Trypsin-MEG	400,000
	Unirradiated Trypsin-MEG	80,000
Dialysis versus Urea	Irradiated Trypsin	500,000
	Irradiated Trypsin-MEG	250,000
	Unirradiated Trypsin-MEG	120,000

denaturing agent is expected to expose the conformationally hindered segments of the protein in which tritium may be trapped and retained. Aliquots (0.5 ml) of the original dialyzed solutions were diluted to 20 ml and dialyzed versus urea. The results of radioactivity counting performed on 1.0 ml aliquots are summarized in Table III.

The results of tritium oxide removal by chromatography and dialysis are similar. Dialysis versus urea is as effective as chromatography in removing exchangeable tritium. The irradiated trypsin samples appear to retain a higher concentration of bound tritium than those samples irradiated in the presence of thiol. Irradiation of the trypsin and trypsin containing thiol in the presence of tritium oxide in comparison with a trypsin sample not exposed to irradiation is consistent with the expected ultraviolet induced formation of free radicals on trypsin. The appreciable retention of activity in the trypsin blank is apparently the result of tritium incorporation in the form of carbon-tritium bonds. This incorporation is apparently induced by the beta rays emitted by the added tritium.

CHAPTER IV

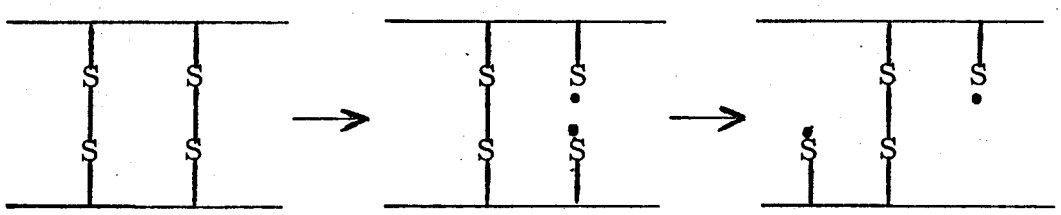
DISCUSSION

Initial studies on the deactivation of trypsin were carried out in a range of pH. The concentration of hydrogen ion was found to have an important effect on the rate of deactivation of trypsin both in the presence and in the absence of thiol. Under the experimental conditions used in this work the greatest degree of protection is observed at pH 4.0. At neutral pH's the sulfhydryl groups produced during irradiation may be spontaneously re-oxidized to disulfide bonds with partial restoration of enzymatic activity (25). This process of enzyme reactivation which may occur in the absence of thiol is a complicating factor when comparing the deactivation of trypsin in the presence of thiol. At pH 3.0, the conformational changes accompanying inactivation appear to be irreversible regardless of whether thiol is present. Further studies were carried out at pH 4.0. It was felt that at this pH complications arising from either trypsin reactivation or digestion occuring at the high pH ranges could be avoided.

The obtained results indicate that the hydrolytic activity of 0.2% and 1.0% trypsin solutions is protected against ultraviolet light induced deactivation by MEG at a concentration of 1×10^{-3} M. Under irradiation conditions resulting in a 50% decrease in trypsin (4.2×10^{-4} M) activity, trypsin containing thiol may retain 58% of the original hydrolytic activity.

A chemical mechanism describing the process involved in trypsin protection from the effects of ultraviolet light by MEG cannot be

substantiated on the basis of currently available evidence. Studies on the inactivation of cystine containing proteins have indicated that thiyl radicals are generated on the protein as a result of the rupture of disulfide bonds (26). In the absence of a protective thiol, the thiyl radical may subsequently lead to disulfide formation with nearby disulfide groups producing a permanent distortion in the protein structure. This is depicted in equation 2:

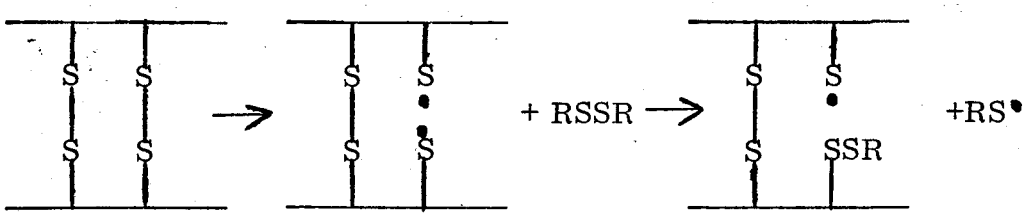


(I) (II) (III)

(Eq. 2)

In this scheme, I represents the intact protein. In structure II the first-formed thiyl radical on the protein is shown to attack a nearby disulfide bond resulting in a protein, III, with modified configuration. A mechanism postulated (23) for the protection of the protein by GED may be schematically represented in equation 3:

(Eq. 3)



(I) (II) (IV)

In the presence of the protective thiol the thiyl radical II may interact with the disulfide bond of GED to form a mixed disulfide, IV. The distortion produced by the formation of a disulfide, IV, may be insufficient to inactivate the enzyme. Furthermore, the reaction outlined in equation 3 may be reversible in vivo and allow reformation of the intact enzyme. In any event the presence of GED could protect the enzyme from the severe structural modification depicted in structure III.

Rapid hydrogen transfer reactions mediated by the thiol may be another possible mechanism by which restoration of the structure and conformational integrity of the irradiated protein could be brought about. High energy irradiation of a polymer (PH) has been known to effect the loss of a hydrogen radical and formation of a macromolecular free radical (P•) (27). The thiol protector may immediately donate a hydrogen and repair the macromolecule before an irreversible change can occur. Formation of the radical P• and the subsequent hydrogen donation leading to the repair of the polymer are depicted in equations 3 and 4:

$$PH \longrightarrow P\bullet + H \qquad \text{(Eq. 3)}$$

$$P\bullet + RSH \longrightarrow PH + RS\bullet \qquad \text{(Eq. 4)}$$

This mechanism may operate during protection of DNA from the gamma-ray induced cross-linking of complimentary strands (28). Electron spin resonance on salmon sperm heads have indicated that protection is accompanied by a hydrogen transfer from the protective thiol, cysteamine,

to the DNA as shown in equation 5:

$$R^{\bullet} + NH_2C_2H_4SH \longrightarrow RH + NH_2C_2H_4S^{\bullet} \quad \text{(Eq. 5)}$$

In addition, thiols have been shown to participate in hydrogen exchange reactions during the protection of benzophenone from the effects of irradiation. The photoreduction of benzophenone in a hydrogen donating solvent is indicated in equation 6:

$$(C_6H_5)_2\,C{=}O + RH \longrightarrow (C_6H_5)_2\,\dot{C}\text{-}OH + R^{\bullet} \quad \text{(Eq. 6)}$$

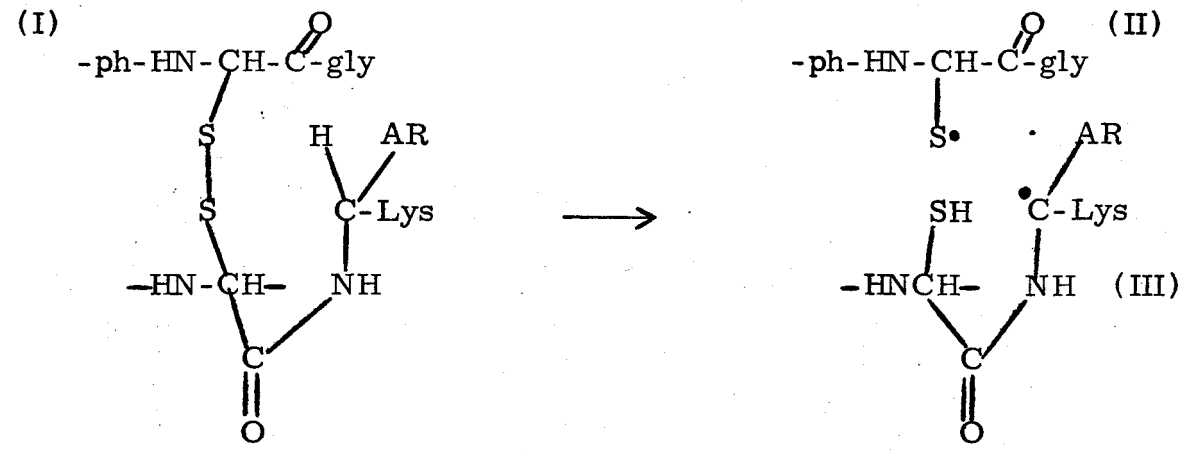
In the presence of mesitylthiol (ASH) however, rapid hydrogen transfer reactions regenerate the starting materials (equations 7 and 8) and the photoreduction is strongly inhibited.

$$(C_6H_5)_2\,\dot{C}\text{-}OH + AS^{\bullet} \longrightarrow (C_6H_5)_2\,C{=}O + ASH \quad \text{(Eq. 7)}$$

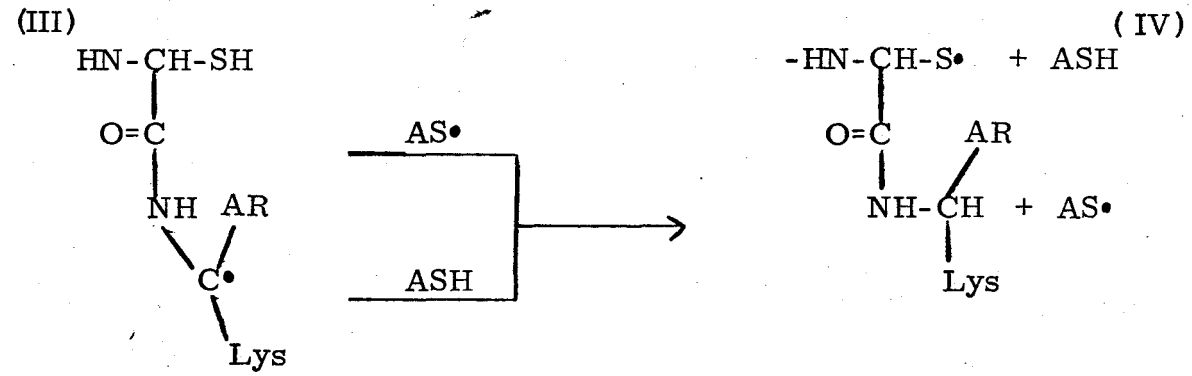
$$R^{\bullet} + ASH \longrightarrow RH + AS^{\bullet} \quad \text{(Eq. 8)}$$

Thiols may act similarly as hydrogen donors towards protein radicals. Disulfide bonds, necessary in maintaining the conformation of proteins, are apparently readily ruptured by ultraviolet light (25). During irradiation of trypsin at 253 mμ energy may be preferentially transferred to the cystine component causing the formation of sulfhydryl groups and thiyl radicals on the protein (29). At this wavelength photochemical energy may be transferred to a tyrosine residue adjacent to a disulfide link via a resonance transfer of energy between aromatic acid residues (30). The excited tyrosine may then transfer hydrogen to the cystinyl component generating a sulfhydryl group on the protein.

This process is depicted in equations 9 and 10 where I represents a section of the trypsin molecule limited by amino acids number 30 and 49 in the amino acid sequence.



(Eq. 9)



(Eq . 10)

An aromatic amino acid, AR, adjacent to a cystine may thus transfer a hydrogen resulting in the rupture of the disulfide bond. In the presence of a hydrogen transfer system such as an appropriate thiol, radical III may rapidly exchange hydrogen giving radical IV with regeneration of the thiol-thiyl radical pair. Radicals IV and II could subsequently combine and

repair the enzyme. The overall effect of these reactions would be the protection of the enzyme from the effects of irradiation.

In the present study we have attempted to determine the degree to which a hydrogen transfer mechanism may operate during the protection of trypsin by AET. Irradiation of the enzyme was carried out in the presence of tritium oxide. The thiol is expected to rapidly exchange hydrogen for tritium as shown in equation 11:

$$ASH + HOT \rightleftharpoons AST + HOH \qquad \text{(Eq. 11)}$$

If the tritiated thiol would participate in repair hydrogen transfers, tritium would be incorporated into the enzyme. As previously described, the tritium would be attached by carbon-tritium bonds and thus would not be exchanged during removal of dialyzable tritium from the enzyme.

The extent of the involvement of a hydrogen transfer mechanism cannot be established on the basis of the data available at this time. The results obtained have indicated that during irradiation of a trypsin solution (1%) leading to 97.5% reduction of the hydrolytic activity of the enzyme, non-exchangeable radioactivity maintained by the enzyme counted at a rate of 500,000 cpm/mg of enzyme. In the presence of MEG a 95.1% loss of enzymatic activity was accompanied by tritium incorporation resulting in 250,000 cpm/mg. A trypsin solution containing MEG and treated similarly but not subjected to irradiation counted at a rate of 120,000 cpm/mg Before these results can be examined in greater detail certain factors

related to the interaction of tritium with proteins should be discussed.

Generally, hydrogen atoms attached to nitrogen, sulfur, and oxygen of a protein are easily exchangeable with protons in solution. Irradiation of a protein in the presence of tritium oxide thus introduces exchangeable tritium which must be removed before an estimation of non-exchangeable carbon-bound tritium can be made.

Separation of the exchangeable tritium attached to ribonuclease from free tritium in solution can be carried out on a chromatographic column packed with Sephadex. Data based on this type of separation indicates that there are at least three classes of exchangeable hydrogens (31). While side-chain hydrogens and peptide hydrogens are easily exchangeable, hydrogens involved in the hydrogen bonding of the α-helix are removed with difficulty. Finally, a third type of hydrogen is extremely difficult to replace. This is probably buried inside the α-helix and removal may be sterically hindered. This is consistent with the observation that all exchangeable tritium can be chromatographically separated from oxidized, denatured, ribonuclease pre-incubated with tritium oxide.

The results indicate that irradiated trypsin and trypsin containing thiol retained significantly greater amounts of carbon-bound tritium than the unirradiated trypsin sample. The increased incorporation is consistent with the expected formation of carbon free radicals on the enzyme during irradiation (32). In addition, tritium incorporation into irradiated

trypsin was found to be greater in the absence of thiol. On the basis of the limited data available at this time it is difficult to assess the significance of the incorporation obtained in the presence of thiol. By appropriate selection of the experimental conditions which would tend to minimize tritium incorporation into trypsin resulting from molecular events not pertinent to protection, more definite conclusions may be reached in the future.

The considerable amount of residual activity maintained by the trypsin blank after dialysis is not surprising. Incorporation of tritium into the enzyme by formation of carbon-tritium bonds may occur spontaneously as a result of the beta radiation emitted by tritium. This type of labeling may account for half of the reported total tritium incorporation during gamma-ray irradiation of ribonuclease (33). Beta labeling thus accounts for the residual non-exchangeable radioactivity of the trypsin blank.

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APPROVAL SHEET

The thesis submitted by Albert J. Iammartino has been read and approved by the thesis director.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

January 20th 1969
Date

Signature of Advisor