

THE INVESTIGATION OF MOLECULAR MECHANISMS IN PHOTODYNAMIC ACTION AND  
RADIOBIOLOGY WITH NANOSECOND FLASH PHOTOLYSIS AND PULSE RADIOLYSIS

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The Investigation of Molecular Mechanisms in Photodynamic  
Action and Radiobiology with Nanosecond Flash  
Photolysis and Pulse Radiolysis

ABSTRACT

Flash photolysis studies have shown that exposed tyrosyl or tryptophyl residues of enzymes are photoionized at approximately the same quantum efficiency as the aqueous amino acids. Comparisons with permanent damage indicate that this primary process contributes to inactivation when a photolyzed aromatic residue is essential or is located adjacent to a catalytic, non-chromoporic residue. Studies on sensitization by the phototherapeutic drug, 8-methoxysoralen (8-MOP), show the triplet state can lead to singlet oxygen generation and that weak binding to poly(dA-dT) suppresses this process. 8-MOP was found to be a potent sensitizer of yeast photoinactivation with negligible post-irradiation recovery. Spin-lable ESR measurements made on yeast cells after photodynamic treatment show that the membrane damage generated by the penetrating dye Toluidine Blue O is more extensive than the external dye, Eosin Y. In related ionizing radiation studies, it was found that 8-MOP is an anoxic radiosensitizer of T7 phage, attributed to enhancement of crosslinks; that complexing of eosin to lysozyme sensitizes the enzyme to oxidizing radicals; that the reactions of  $\text{Br}_2^-$  and  $(\text{CNS})_2^-$  with tryptophan generate the same initial radical as UV-excited photoionization.

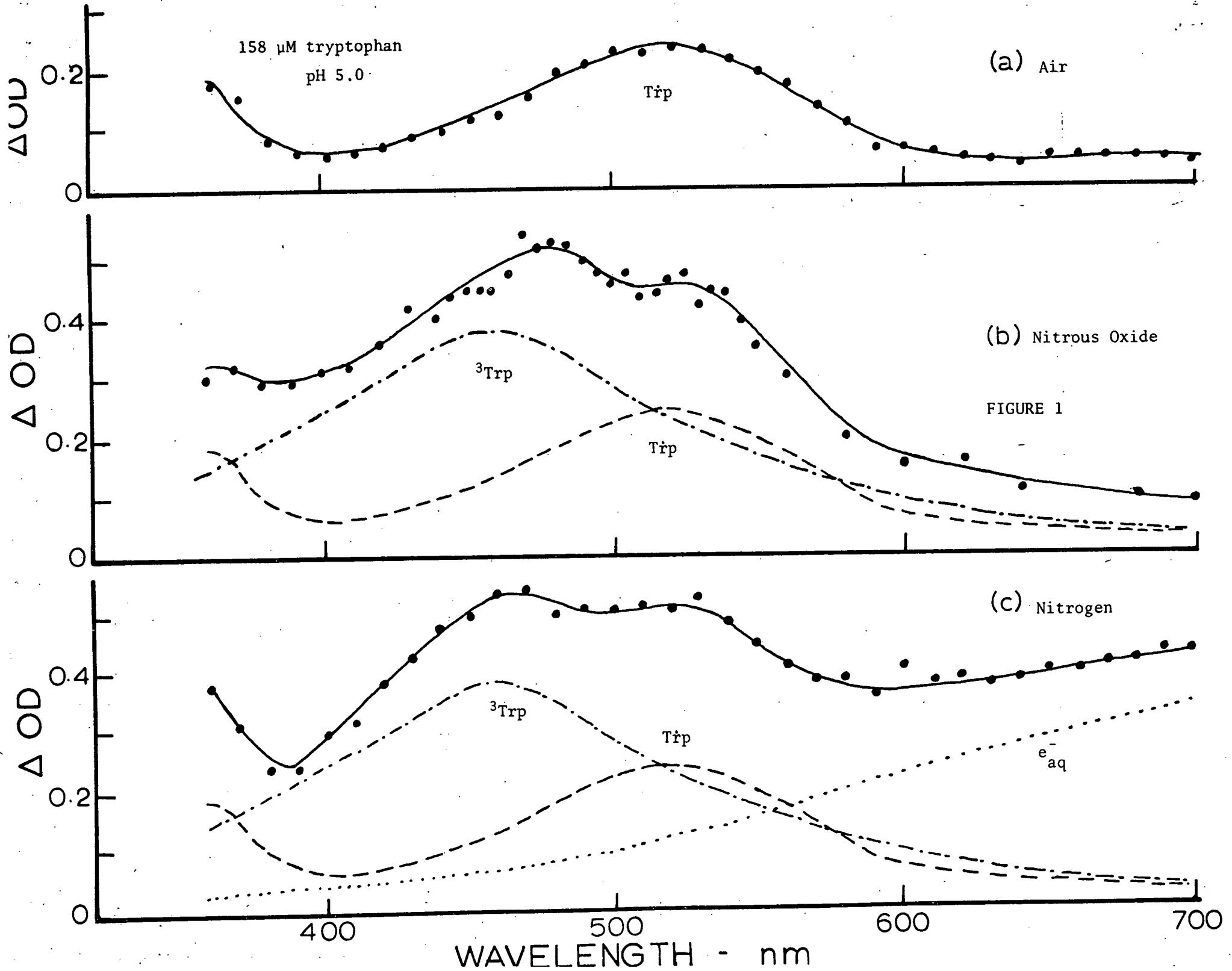
### 1. Flash Photolysis of Enzymes

Flash photolysis studies on the following enzymes in aqueous solution have been carried out in this laboratory with the objective of identifying the initial photochemical reactions and relating them to permanent damage: lysozyme (Grossweiner and Usui, 1971), ribonuclease A (Volkert and Grossweiner, 1973), trypsin (Kaluskar and Grossweiner, 1974), and papain (Baugher and Grossweiner, 1975). During the course of this work new information has become available on photoionization of aromatic amino acids, particularly the electron quantum efficiencies and extinction coefficients of the product radicals, which makes possible a more detailed analysis. This report summarizes a re-investigation of these enzymes plus new work on subtilisin Carlsberg, utilizing improved experimental and analytical techniques.

The flash photolysis spectra were taken with an apparatus using a 20  $\mu$ sec photolysis flash provided by two xenon flash lamps operated at 225 joules input per lamp. The flash lamps were located along the axes of an elliptical reflector and parallel to the 20 cm long, quartz irradiation cell. The cell diameter was 1.3 cm with a 1 cm coaxial outer chamber which contained glacial acetic acid as a filter for  $\lambda < 250$  nm. The 10  $\mu$ sec spectroflash was obtained with a capillary, xenon lamp operated at 65 joules input and focused through the cell into a Bausch and Lomb 'medium' quartz prism spectrograph. All spectra were taken at 5  $\mu$ sec peak-to-peak time delay on 10 in., Kodak 103F spectroscopic plates and scanned with a Baird-Atomic SD-1 microdensitometer. A neutral filter was photographed on each plate to check the sensitivity calibration, and a test experiment with aqueous tryptophan was run every five experiments to ensure proper operation of the entire system. Each experiment was repeated ab initio at least four times, and the transient spectra were

## FIGURES 1 - 7

Xenon lamp flash photolysis spectra taken at 5  $\mu$ sec time delay on Kodak Type 103-F spectroscopic plates. The component spectra are:  
-x-x-x-x-x- tyrosyl radical (Tyr) , - - - - - tryptophanyl radical (Trp) ,  
o o o o o cystyl electron adduct (-S:S-) , . . . . . hydrated electron  
( $e^-_{aq}$ ) , -.-.-.-.- tryptophan triplet state ( $^3$ Trp) Figs. 1,2,4,5,7  
and tyrosyl triplet state ( $^3$ Tyr) Figs. 3,6. The solid lines are the  
sum of the contributions as fitted to the experimental spectra o .



computed at 5 nm intervals from 340 nm to 700 nm. The resolution into component spectra was carried out by fitting at 340 nm, 420 nm, 460 nm, 520 nm, and 700 nm. Typically, four or five iterations were required for a satisfactory fit where the calculated overall absorption fell within the standard deviation. The aromatic amino-acid radical spectra were obtained by flash photolysis of aqueous tyrosine and tryptophan (e.g. Figure 1 (a)). Published results were used for the hydrated electron (Fielden and Hart, 1967) and the disulphide bond electron adduct (Adams et al., 1967).

The new data consist of flash photolysis spectra taken of the enzymes and corresponding mixtures of the chromophores: tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), cystine (Cys) and glycyl-glycine to account for the absorption of the peptide bonds. The mixtures had the same integrated absorption as the enzyme for  $\lambda > 250$  nm to within 5 per cent, with a 3-5 nm red shift in the 280 nm band, which does not alter the analysis of the results. Measurements were made with air and  $N_2O$  present to identify the contributions of aromatic triplet states and hydrated electrons. The experimental approach can be illustrated by considering flash spectra obtained with aqueous Trp and Tyr and the effects of adding Cys. The transient absorption at 510 nm and 330 nm in Figure 1 (a) obtained with Trp in air has been identified with the neutral tryptophanyl radical Trp generated by electron ejection at the indole ring N-H bond followed by rapid deprotonation, and the additional band near 460 nm in  $N_2O$  (Figure 1 (b)) was attributed to the tryptophan triplet state  $^3Trp$  (Santus and Grossweiner, 1972). The  $^3Trp$  band is resolved in  $N_2$  (Figure 1 (c)) as well as the broad  $e^-_{aq}$  absorption peaking near 720 nm. The photoionization quantum yields at the band maxima, as measured with laser flash photolysis, are given in Table I, and the reported values of the radical extinction coefficients are summarized in Table II. The actinometry of

Table I  
Electron quantum yields for aqueous amino acids

<u>Amino Acid</u>	<u>pH</u>	$\phi_{e^-}$ <sup>(1)</sup>	<u>Reference</u>
Tryptophan	6.0	0.080	Bent and Hayon (1975 b)
	11.0	0.210	" "
	aq 9.0 11.8	0.250	Bryant et al. (1975)
		0.360	" "
		0.430	" "
	aq	0.11 (2)	This Work
Tyrosine	7.5	0.095	Bent and Hayon (1975 a)
	11.5	0.150	" "
	aq 9.0	0.290	Bryant et al. (1975)
		0.310	" "
	aq	0.071 (3)	This Work
Phenylalanine	7.5	0.034	Bent and Hayon (1975 c)
	11.1	0.028	" "

(1) electron quantum yield at 265 nm by laser flash photolysis

(2)  $[e^-_{aq}]_0 = 1-5 \mu M$

(3)  $[e^-_{aq}]_0 = 3 \mu M$

Table II  
Properties of Amino Acid Radicals

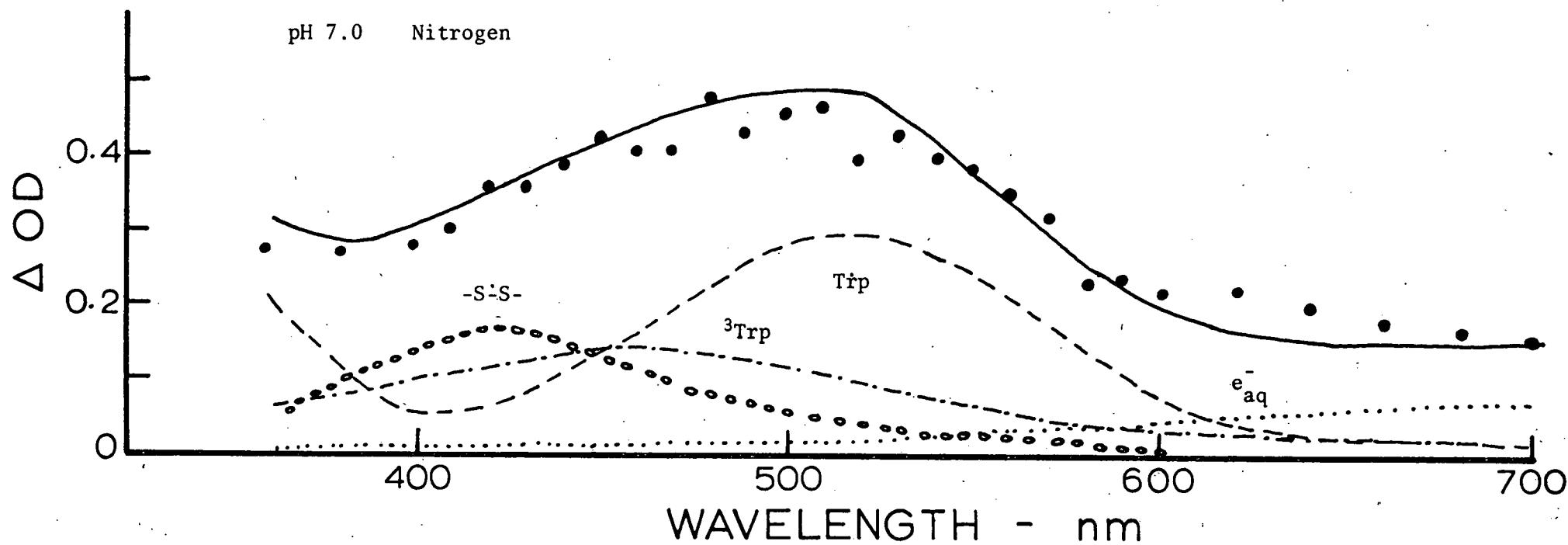
<u>Solute</u>	<u>Radical</u>	<u>Spectra</u> <sup>(1)</sup>	<u>Reference</u>	
Tryptophan				
pH 7-12	Trp	510 nm 1800±200	330 nm 3100±300	Bryant et al. (1975)
pH 7		510 nm 1750(±20%)	320 nm 2800(±20%)	Redpath et al. (1975)
pH 7		520 nm 1840	320 nm 2860	Posner et al. (1975)
Tyrosine				
pH 7	Tyr	410 nm 2600±500	Bryant et al. (1975)	
pH 7		410 nm 2750±200	Feitelson and Hayon (1973)	
1-Methyltryptophan				
pH 7	1-MeTrp <sup>+</sup>	570 nm 2600(±20%)	340 nm 3900(±20%)	Redpath et al. (1975)
pH 7		580 nm 2900±600	Bryant et al. (1975)	
Cystamine				
pH 7.3	RSSR	410 nm 9400	Adams et al. (1969a)	
pH 7.7		415 nm 9000	Hoffman and Hayon (1972)	
Cystine				
pH 7.7	RSSR	420 nm 58800	Hoffman and Hayon (1972)	

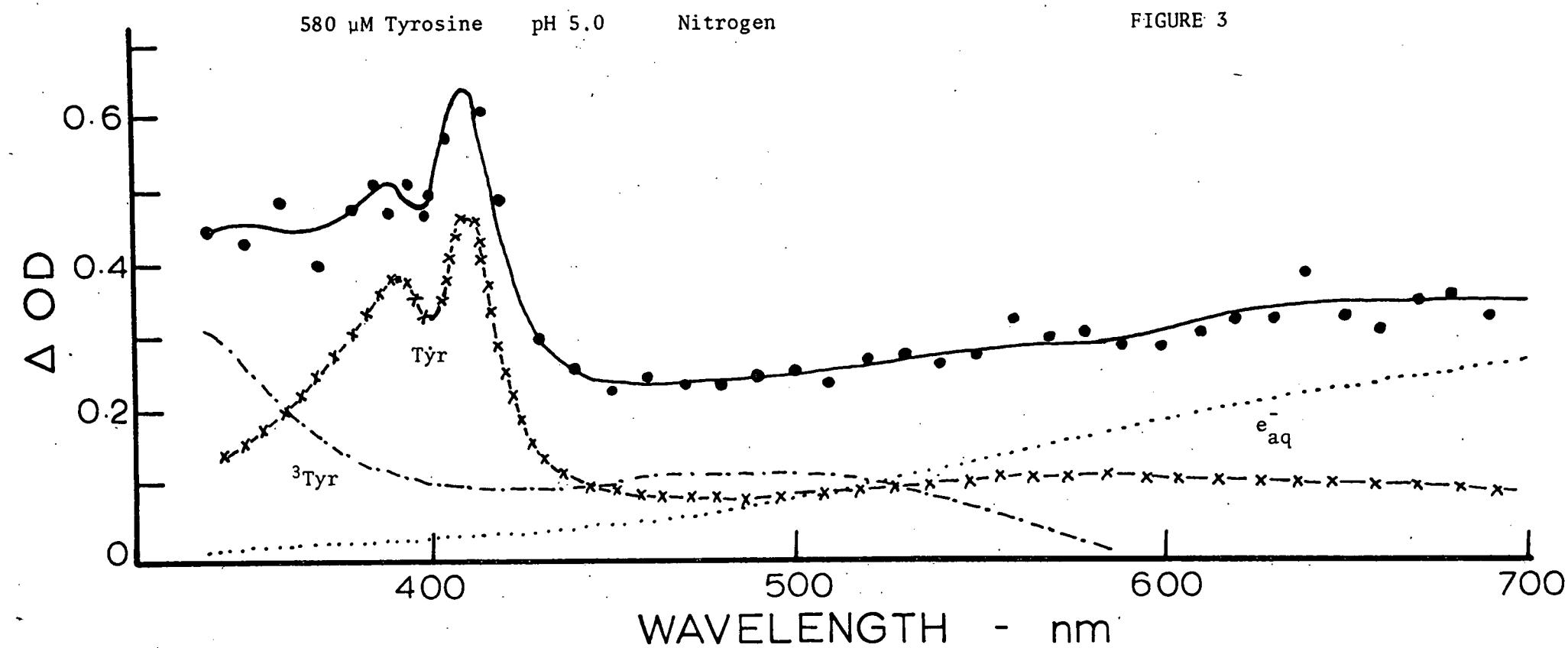
(1) upper, absorption maxima; lower, extinction coefficient (liters/mole-cm)

122  $\mu$ M Tryptophan + 170  $\mu$ M Cystine

pH 7.0      Nitrogen

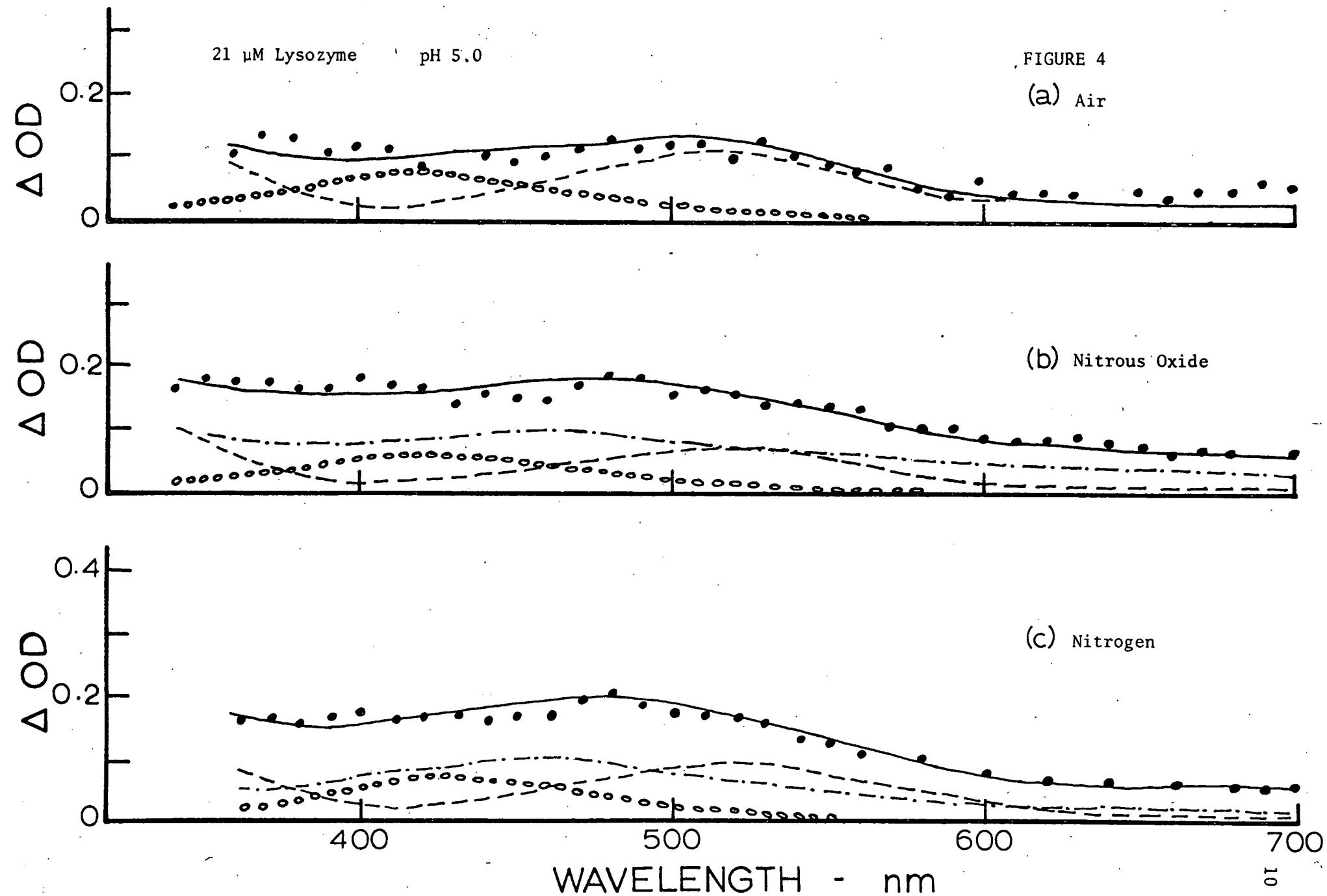
FIGURE 2

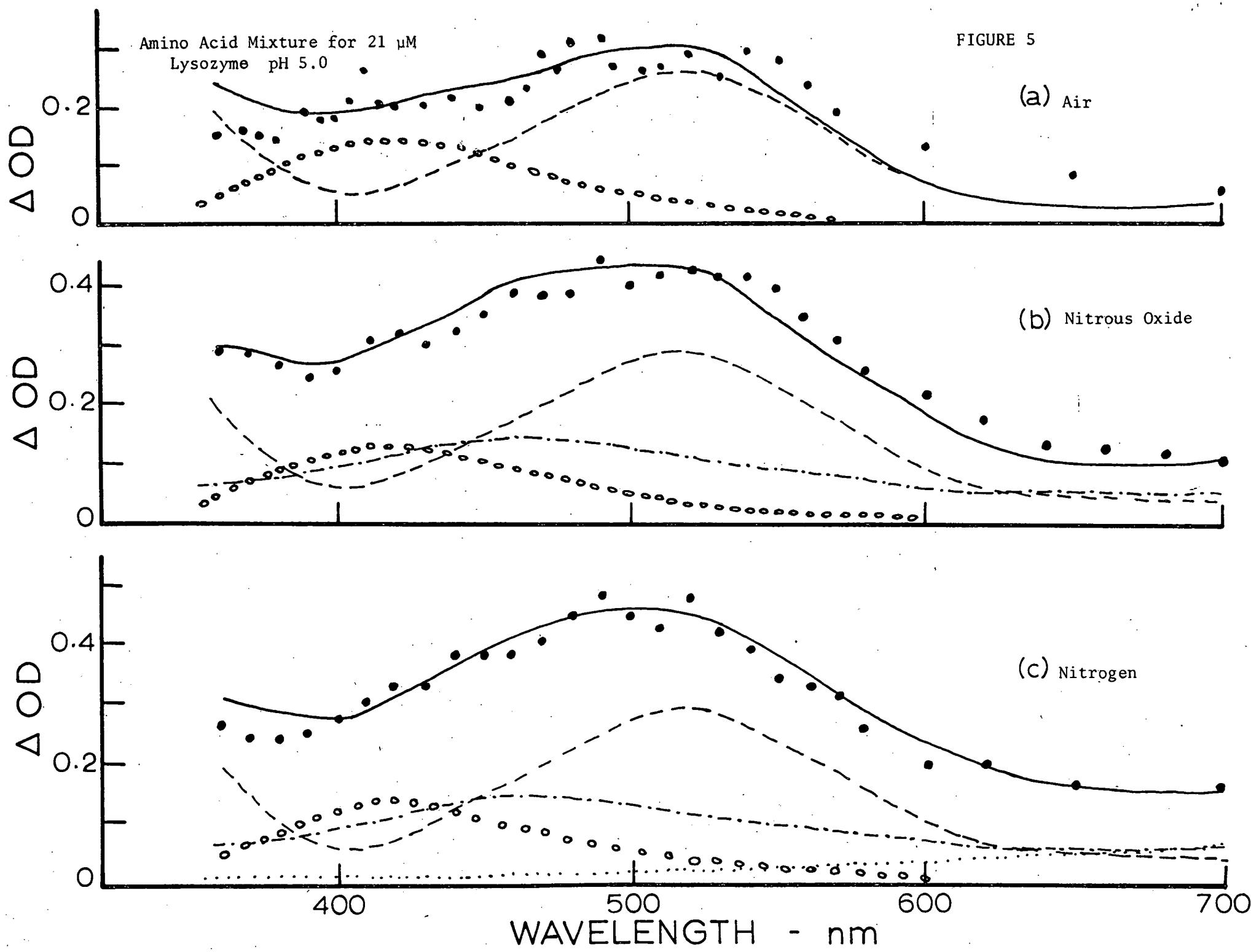




Bent and Hayon (1975a,b,c) was based on the T-T absorption of anthracene in cyclohexane. Bryant et al. (1975) measured electron yields relative to  $\text{Fe}(\text{CN})_6^{4-}$  taking  $\phi_{e^-}$  (265 nm) = 0.52 based on Shirom and Stein (1971). The new work has shown that the initial electron yield from aqueous Trp is a linear function of the laser intensity up to 15  $\mu\text{M}$  (per pulse), but the electron yield from  $\text{Fe}(\text{CN})_6^{4-}$  saturates above 30  $\mu\text{M}$ . The  $\text{Fe}(\text{CN})_6^{4-}$  actinometer was employed for Trp and Tyr photoionization, based on initial electron yields of 1-5  $\mu\text{M}$  comparable to the present xenon-lamp experiments. The laser-flash experiments on aqueous Trp (Bryant et al., 1975; Bazin et al., 1975) show that the electron and the Trp radical disappear via a first-order process of 0.7-0.8  $\mu\text{sec}$  lifetime, attributed by Bryant et al. (1975) to the formation of a short-lived complex between the radical and  $e^-_{\text{aq}}$  in which the back reaction competes with dissociation into the free species. Subsequently, the radical is long-lived, while the electron continues to decay by fast, second-order reactions. The flash spectra obtained at 5  $\mu\text{sec}$  delay in the present work should provide reliable values of the Trp yields after the initial recombination stage.

The irradiation of Trp in the presence of Cys (Figure 2) leads to the 420 nm absorption of the disulphide electron adduct RSSR. The comparison with Figure 1(c) shows that both  $e^-_{\text{aq}}$  and  $^3\text{Trp}$  are diminished relative to Trp indicating that  $e^-_{\text{aq}}$  capture by Cys and reaction of  $^3\text{Trp}$  with Cys contribute to RSSR formation. The RSSR yield was about one-third lower in air accompanied by the quenching of  $^3\text{Trp}$  and unchanged in  $\text{N}_2\text{O}$  where  $^3\text{Trp}$  was not quenched. The flash spectrum of Tyr in Figure 3 shows the characteristic 410 nm and 390 nm bands of the phenoxy-type tyrosyl radical Tyr (Grossweiner et al., 1963). The spectrum shows also  $e^-_{\text{aq}}$  and bands near 295 nm and 575 nm attributed to the tyrosine triplet state  $^3\text{Tyr}$  by Bent and Hayon (1975a). The addition of air led to the quenching of  $^3\text{Tyr}$  and  $e^-_{\text{aq}}$  and no change in Tyr.





The flash spectra of lysozyme in Figure 4 show transients corresponding to Trp at 510 nm, -S-S- at 420 nm and a detectable  $^3\text{Trp}$  yield at 460 nm. The small effect of air or  $\text{N}_2\text{O}$  on the -S-S absorption suggests that the electron transfer from Trp residues to disulfide bridges takes place internally. However, the data do not distinguish between a fast intramolecular process and the type of short-lived intermediate complex formation postulated for the aqueous amino-acid systems. The amino-acid mixture corresponding to lysozyme gave similar flash spectra with higher yields of transient products (Figure 5). The dependence on air and  $\text{N}_2\text{O}$  are equivalent to the enzyme. The ratio of the Trp yield in an enzyme to the yield in the corresponding amino acid mixture ( $\gamma_{\text{Trp}}$ ) leads to two parameters characterizing the photolysis of Trp residues. Since the amino-acid mixture has essentially the same absorbance for  $\lambda > 250$  nm, multiplying  $\gamma_{\text{Trp}}$  by 0.11 the photoionization efficiency of aqueous Trp (Table I) gives the average photoionization efficiency of Trp residues in the enzyme based on light absorbed only by Trp residues ( $\phi'_{\text{Trp}}$ ). (The amino-acid mixture acts as a secondary actinometer that corrects for light absorbed by other chromophores.) Multiplying  $\gamma_{\text{Trp}}$  by the number of tryptophyl residues ( $n_{\text{Trp}}$ ) gives a quantity  $\Delta n_{\text{Trp}}$  that can be considered as the number of 'photolabile' Trp residues, i.e. the number of residues photoionized at the same quantum efficiency as aqueous Trp, assuming that the other Trp residues in the enzyme are not photolyzed. In the case of lysozyme  $\Delta n_{\text{Trp}} = 2.5$ . Since inactivation quantum yields and residue destruction yields usually are reported in terms of light absorbed by the entire enzyme, it is convenient to convert  $\phi'_{\text{Trp}}$  to this basis by multiplying by the fraction of light absorbed by Trp residues ( $f_{\text{Trp}}$ ). This quantity has been calculated for each enzyme by numerical integration of the amino-acid absorptions, assuming that the flash-lamp spectral distribution is constant from 250 nm to 320 nm. For

FIGURE 6

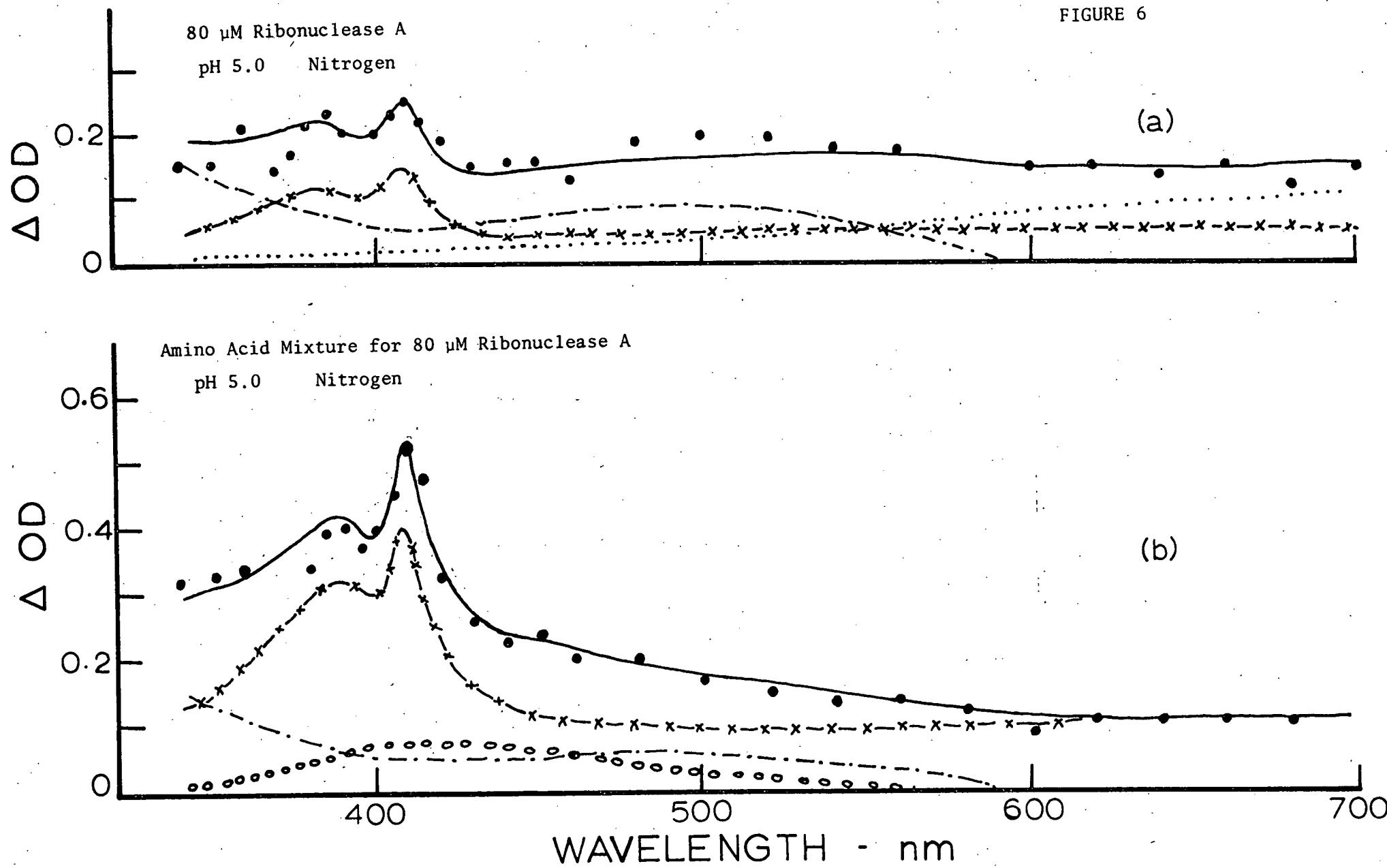
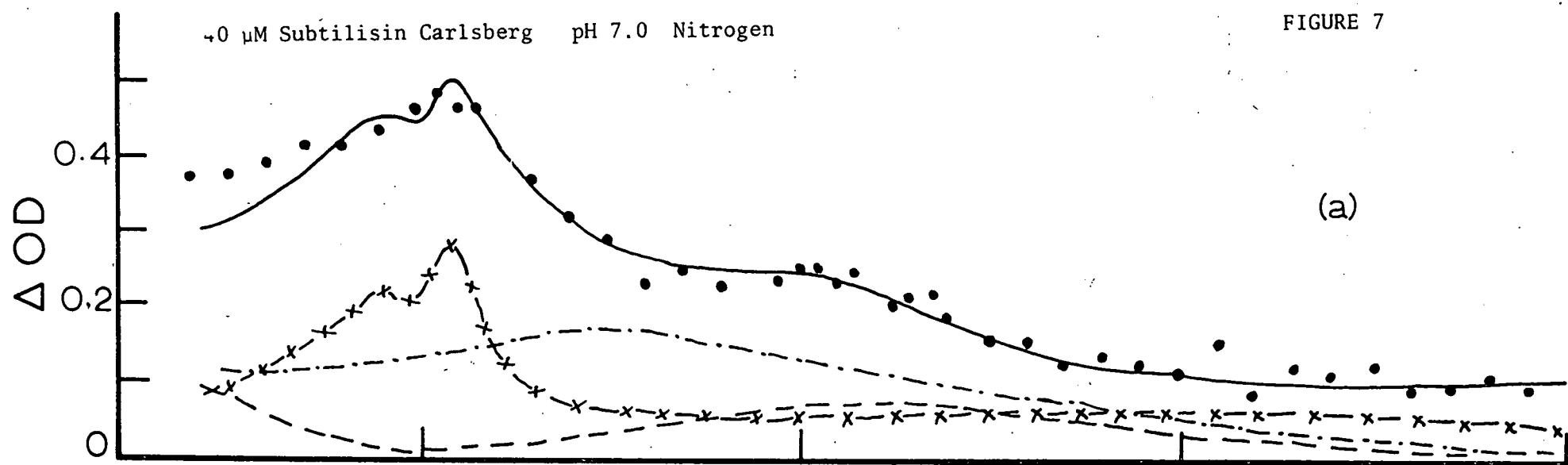


FIGURE 7

40  $\mu$ M Subtilisin Carlsberg pH 7.0 Nitrogen



Amino Acid Mixture for 40  $\mu$ M Subtilisin Carlsberg  
pH 7.0 Nitrogen

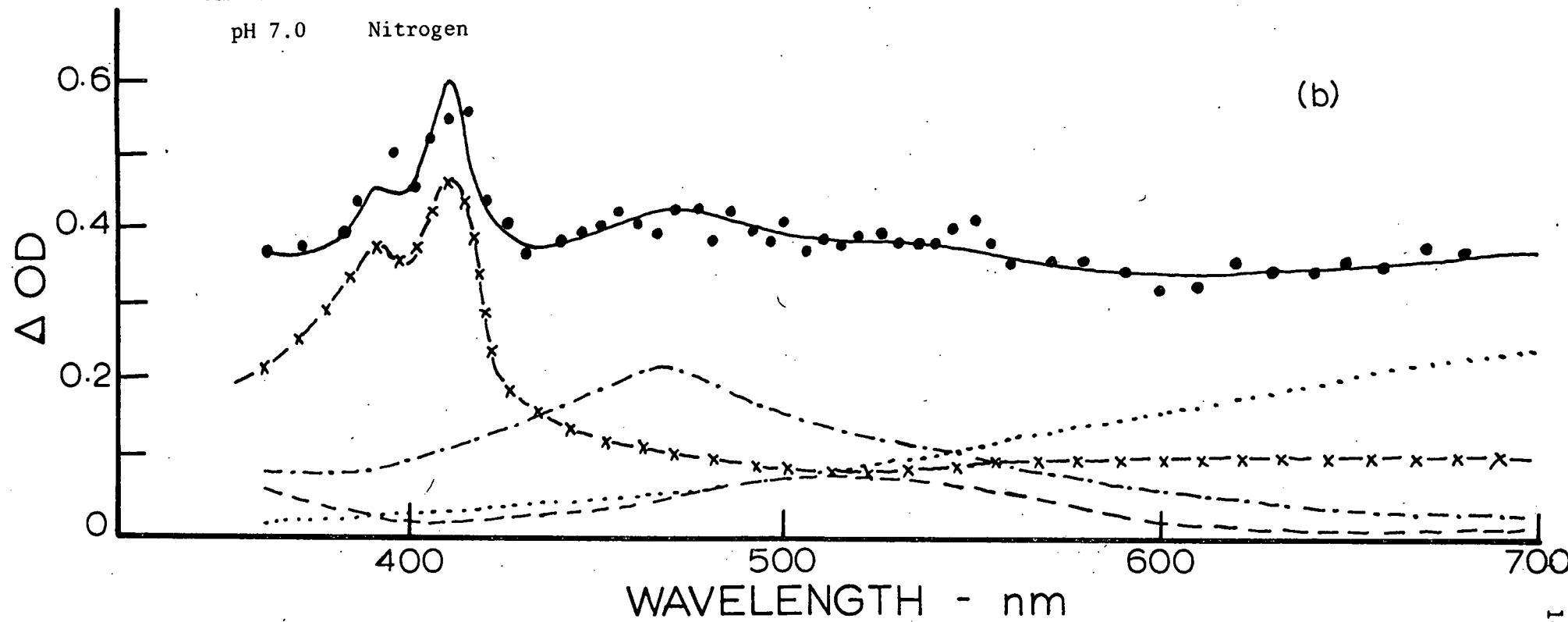


Table III  
Summary of flash photolysis results

<u>Enzyme</u>	pH	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
		$\gamma_{Trp}$	$n_{Trp}$	$\Delta n_{Trp}$	$\bar{\phi}_{Trp}$	$f_{Trp}$	$\bar{\phi}_{Trp}$	$\gamma_{Tyr}$	$n_{Tyr}$	$\Delta n_{Tyr}$	$\bar{\phi}_{Tyr}$	$f_{Tyr}$	$\bar{\phi}_{Tyr}$	$\bar{\phi}_{-s-s-}$
Lysozyme	5.0	0.42	6	2.5	0.046	0.90	0.042	--	3	--	--	0.07	--	0.005
Trypsin	4.0	0.55	4	2.2	0.060	0.68	0.041	--	10	--	--	0.24	--	0.005
Papain	7.0	0.59	5	3.0	0.065	0.62	0.040	--	16	--	--	0.35	--	0.007
Subtilisin Carlsberg	7.0	1.00	1	1.0	0.11	0.14	0.015	0.60	13	7.8	0.042	0.86	0.036	0.000
Ribonuclease A	5.0	--	0	--	--	--	--	0.40	6	2.4	0.028	0.80	0.022	0.000
	11.8	--	0	--	--	--	--	0.58	6	3.5	~0.09	~0.85	~0.07	0.000

Col. 1 Trp yield in enzyme : Trp yield in amino acid mixture

Col. 2 number of Trp residues in enzyme

Col. 3 Col. 1  $\times$  Col. 2

Col. 4 quantum yield of Trp formation based on Trp residue absorption

Col. 5 fractional absorption by Trp residues in enzyme

Col. 6 Col. 4  $\times$  Col. 5; quantum yield of Trp formation based on enzyme absorption

Col. 7 - Col. 12 equivalent to Col. 1 - Col. 6 for Tyr residues

Col. 13 quantum yield of -s-s- formation based on enzyme absorption

lysozyme  $f_{Trp} = 0.90$ , leading to  $\bar{\phi}_{Trp} = 0.046$  as the average photoionization efficiency of Trp residues. The initial yield of the disulfide bridge electron adduct ( $\phi_{-S-S-}$ ) has been estimated as 0.005 in lysozyme by comparing the Trp and RSSR transients on the spectral plates ( $N_2$ -saturated) taking the ratio of the extinction coefficient in Table II.

The transient spectra for trypsin and papain were reported in recent publications (Kaluskar and Grossweiner, 1974; Baugher and Grossweiner, 1975). The measurements were repeated leading to the results in Table III. The only essential difference from the earlier work was the resolution of  $^3Trp$  in trypsin which was not quenched by air, in contrast to papain and lysozyme. New transient spectra for RNase A and the amino-acid mixture are given in Figure 6. In contrast to the earlier work which reported that Tyr residues are photoionized only in alkaline solutions (Volkert and Grossweiner, 1973), the present results show a definite yield of Tyr at pH 5.0 which is enhanced by 50 per cent at pH 11.8. The broad shoulder on the short wave-length side of the 390 nm Tyr band is attributed to the  $^3Tyr$  absorption. The triplet state was quenched by air in the mixture but not in the enzyme. The presence of air and  $N_2O$  had a negligible effect on the Tyr bands in RNase at pH 5.0 and pH 11.8. A low yield of the disulfide electron adduct was resolved in the mixture but not in the enzyme. The flash spectra for 40  $\mu M$  subtilisin Carlsberg at pH 7.0 in Figure 7 indicate that both Trp and Tyr residues are photolysed. The  $^3Trp$  and  $^3Tyr$  bands are resolved and were quenched by air. The high value of  $\gamma_{Trp}$  indicates that the single Trp residue in the enzyme is photoionized at about the same efficiency as aqueous Trp.

The flash photolysis results show that the photoionizations of Trp and/or Tyr residues are important initial photochemical reactions. In addition to the enzymes reported here, Trp and Tyr were identified in human

Table IV  
Correlation of flash photolysis yields with residue exposure

<u>Enzyme</u>	(1) $n_{Trp}^{exp}$	(2) $\Delta n_{Trp}$
Lysozyme	~2 (a)	2.5
Trypsin	2.4 (b)	2.2
Papain	2.2 (c)	3.0
Subtilisin Carlsberg	0.8 (d)	1.0
Ribonuclease A	~2 (e) (f)	2.4 (e)

Col. 1 number of exposed tryptophyl residues in enzyme  
 Col. 2 photolabile tryptophyl residues from Table III

- (a) Imoto et al. (1972)
- (b) Villanueva and Herskovits (1971)
- (c) Weinryb and Steiner (1970)
- (d) Markland and Smith (1971)
- (e) For tyrosyl residues
- (f) Herskovits and Laskowski (1968)

serum albumin (1 Trp, 18 Tyr, 8 Cys) and bovine carbonic anhydrase (8 Trp, 7 Tyr, 0 Cys) and Tyr was observed in calf thymus histone (0 Trp, 4 Tyr, 0 Cys) (Kaluskar, 1973). In every case  $e^-_{aq}$  has been identified in the absence of electron scavengers, and the disulphide bridge electron adduct was resolved in each protein containing Cys except RNase A. In evaluating the role of aromatic residue photolysis on the activity it is assumed that radical back reactions prior to 5  $\mu$ sec and short-lived excited states do not influence the permanent damage. However, the possibility that such reversible processes may lead to subtle changes in the active conformation is not discounted. Another deficiency is the absence of direct information about the role of the weakly absorbing residues. In the present work, it is assumed that photolysis of an aromatic residue immediately adjacent to an essential residue on the primary chain or in close proximity in the spatial structure may lead to inactivating damage. Direct photolysis of disulfide bridges is considered in enzymes where Cys absorption is significant. The calculation of  $\Delta n_{Trp}$  or  $\Delta n_{Tyr}$  is based on the assumption that the aromatic residues can be divided into two groups, the 'photolabile' residues that photoionize at high efficiency and those which are relatively stable. This distinction is supported by the correlation between the  $\Delta n$ 's and the number of exposed aromatic residues as determined with conventional methods (Table IV). In the case of subtilisin Carlsberg, where both Trp and Tyr are photoionized, the estimate of 8.8 exposed Tyr residues (Markland and Smith 1971) is in good agreement also with  $\Delta n_{Tyr} = 8$ . The quenching of  $^3Trp$  or  $^3Tyr$  by air in lysozyme, papain and subtilisin, but not in RNase or trypsin, may be related to exposure also.

The flash-photolysis results have been related to the enzyme structure and permanent damage in order to specify probable inactivation mechanisms

for each enzyme. Supporting data on residue sensitivities have been taken from pulse radiolytic and photodynamic studies when available. Our evaluation is as follows:

Hen lysozyme. The major initial photochemical reaction deduced from the flash photolysis results is the photoionization of 2 to 3 Trp residues. The independence of  $\phi_{in}$  of pH or the presence of air indicates that ejected hydrated electrons do not induce inactivation, in agreement with the work of Adams et al. (1969b) showing that  $e^-_{aq}$  generated by pulse radiolysis does not inactivate lysozyme. Photochemical inactivation is accompanied by almost complete loss of lysozyme fluorescence, but with the destruction of only two Trp residues (Grossweiner and Kepka, 1973). This result can be explained by the work of Imoto et al. (1971) showing that Trp 62 and Trp 108 account for about 90 per cent of lysozyme fluorescence, if it is assumed that the same residues are preferentially photoionized. Since Trp 62, Trp 63, and Trp 108 are essential, this reaction accounts also for the loss of lytic activity. The insensitivity of the -S-S- band to air or  $N_2O$  may be a consequence if short-range electron transfer from Trp 63 to adjacent Cys 64-80. The flash-photolysis mechanism is consistent with pulse radiolysis experiments of Adams et al. (1969b), showing that Trp 108 is the primary inactivating target of OH radical attack and also the work of Kepka and Grossweiner (1973), showing that singlet oxygen, generated by eosin sensitization, inactivate lysozyme by reacting with Trp 108. Furthermore, Jori, et al. (1974) have shown that Trp 63 and Trp 108 are the sensitive targets when lysozyme inactivation is photosensitized by proflavin.

Papain. This enzyme is unusual in that irradiation of the original preparation (under  $N_2$ ) leads first to activation and then to inactivation. Photochemical activation has been identified with the formation of one sulphhydryl group per molecule (Dose and Risi, 1972) by reduction of the mixed

disulphide at cysteinyl site 25. The flash spectra indicate that about 3 Trp residues are photolabile. According to the X-ray structure only Trp 69 is located on the enzyme surface (Drenth et al., 1968). However, the photo-dynamic inactivation studies of Jori and Galianzo (1971) indicate that Trp 7 and Trp 177 are partially exposed in solution and Trp 177 was shown to be essential. The side-chain of Trp 177 is in contact with His 159 in the active site, suggesting that photoionization of this residue is the photo-chemical inactivating step. The pulse radiolysis investigation of papain by Adams and Redpath (1974) also led to the conclusion that papain contains at least one essential Trp residue. The parallel to the photochemical case is apt because the reactive radical anions employed,  $(\text{CNS})_2^-$  and  $\text{Br}_2^-$  oxidize Trp to the same Trp radical as formed by photoionization (Redpath et al., 1975).

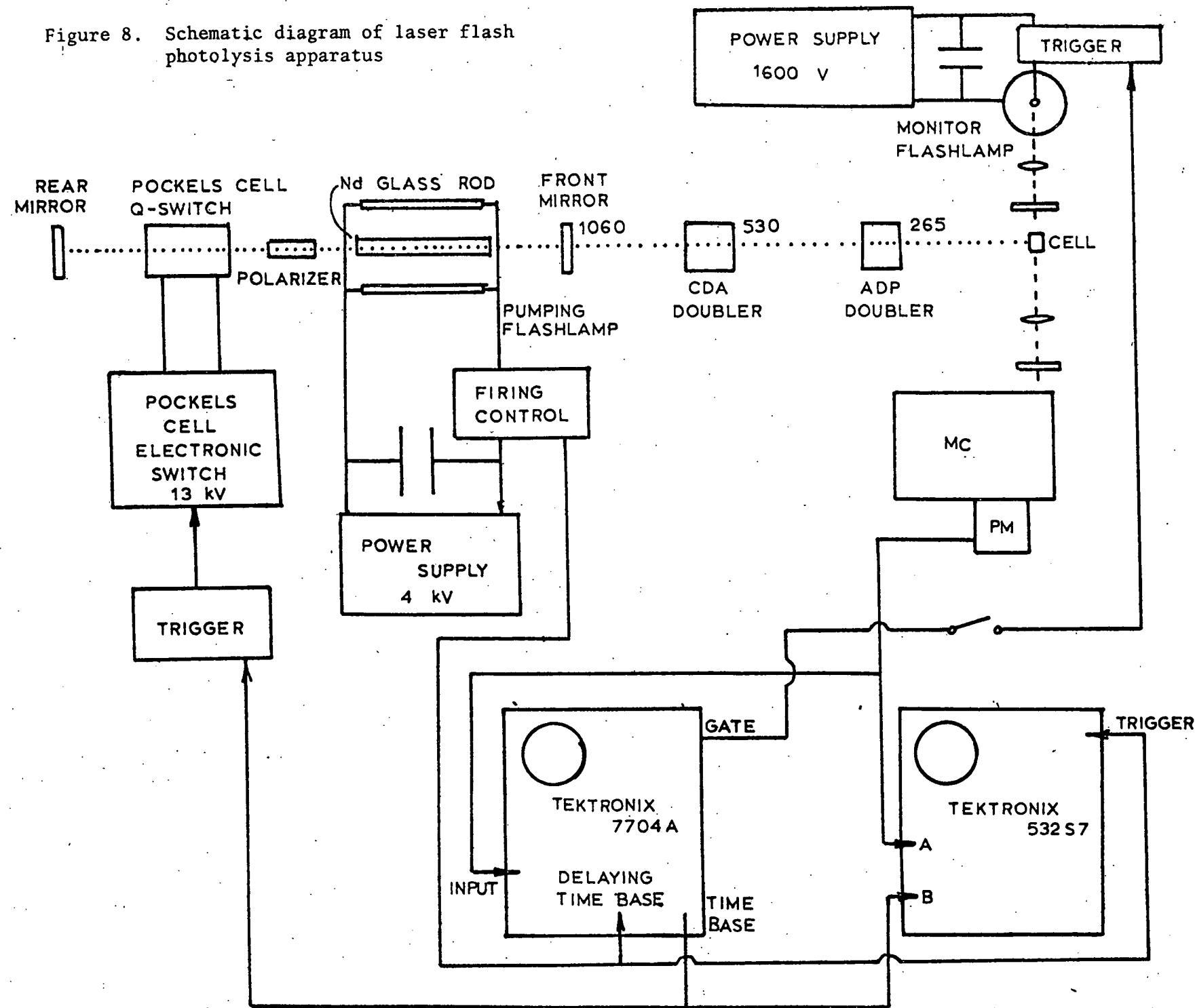
Trypsin. The flash spectra show that 2 to 3 Trp residues are photolabile. Trp 199 located adjacent to the key catalytic site Ser 198 is a likely UV target. Volkert and Ghiron (1973) found that the quantum yields of inactivation and Trp destruction are the same at 280 nm, suggesting that only one Trp residue is essential. The formation of -S-S- via internal electron transfer may involve Trp 199 near Cys 179-203 or Trp 127 near Cys 13-143 in the tertiary structure. Tyr 48 is adjacent to Cys 31-47 on the primary chain, but the absence of Tyr in the flash spectra argues against this electron transfer. Action spectra (Kaluskar and Grossweiner, 1974) indicate that light absorbed by Cys and Tyr residues also contributes to  $\phi_{in}$ . The direct photolysis of Cys 31-47 and/or its sensitized photolysis by Tyr 48 are reasonable pathways, since essential His 46 is adjacent to this disulphide bridge.

Ribonuclease A. The principal aromatic chromophores are the 6 Tyr residues, none of which are believed to be essential. The flash spectra show that 2 or 3 Tyr residues are photolabile, which should include the exposed

residues Tyr 73, Tyr 76, and Tyr 115. The internal electron transfer from buried Tyr 25 to Cys 26-84 might contribute to Tyr formation and  $\phi_{in}$ . Light absorption by Cys is relatively high in RNase (17.6 per cent for flash irradiation) and direct photolysis of essential Cys 26-84 or Cys 40-95 also should be inactivating. The small yield of permanent Tyr photolysis indicates that the efficient photoionization reaction leading to  $e_{aq}^-$  formation is not an inactivating process. This conclusion is supported by action spectra (Setlow and Doyle, 1957) showing that  $\phi_{in}$  is minimal at 295 nm in alkaline solutions where the Tyr initial yield is high.

Subtilisin Carlsberg. The production of both Trp and Tyr by flash photolysis reflects the 13 Tyr to 1 Trp composition. (Both radicals were observed also in human serum albumin with 18 Tyr and 1 Trp residues (Kaluskar 1973).) The relative yields indicate that the single Trp residue at site 113 and about 8 Tyr residues are photolabile. No aromatic residue is located near the essential residues Asp 32, His 64, and Ser 221 and the enzyme contains no sulphur. The biochemical data do not reveal Trp sensitivity in subtilisin (Markland and Smith, 1971). However, pulse radiolysis studies (Bisby, 1975) show that  $Br_2^-$  reactions with His and  $(CNS)_2^-$  reactions with Trp contribute to inactivation. The marked dependence of  $\phi_{in}$  on oxygen was observed previously in bovine carbonic anhydrase by Walrant and Santus (1974a,b), who demonstrated that the Trp photooxidation product N-formylkynurenone photosensitizes the production of singlet oxygen, which in turn attacks His residues. The role of Trp or Tyr photolysis in the anaerobic process is not apparent. One possibility is the internal attack of the ejected photoelectron on an essential His residue. The occurrence of oxygen sensitivity in two enzymes without disulfide bridges suggests that the capture of ejected electrons at this site competes with the production of damaging  $O_2^-$  when cystine is present.

Figure 8. Schematic diagram of laser flash photolysis apparatus



## 2. Laser Flash Photolysis of Tryptophan

A detailed investigation on the ultraviolet laser flash photolysis of tryptophan has been in progress with the objective of determining the basic photochemistry. The role of tryptophan as a major chromophore makes it an important target in the ultraviolet photolysis of proteins, in addition to the relevance for aromatic photochemistry in general.

The investigation is being carried out with a 300 megawatt Holobeam, Q-switched Nd:glass laser providing a 17-nsec pulse. The experimental arrangement for flash photolysis is shown in Figure 8. The 1060 nm beam from the laser is doubled by a CDA crystal and then quadrupled by an ADP crystal to produce a 60 mJ output pulse at 265 nm. The transient species are monitored by a xenon flashlamp energized by a 200 mfd capacitor charged to 1600 volts to provide a 300 microsecond flash. A timing circuit fires the laser during the peak flat output of the xenon flashlamp. The transient spectra are determined with a grating monochromator with a photomultiplier detector at the exit slit and recorded on an oscilloscope.

As noted above, xenon flash photolysis studies (Santus and Grossweiner, 1972) demonstrated that the primary photoproducts in the ultraviolet irradiation of tryptophan are the hydrated electron  $e_{aq}^-$  ( $\lambda_{max}$  720 nm), the neutral tryptophanyl radical  $Trp$  ( $\lambda_{max}$  520 nm), and the triplet state of tryptophan  $^3Trp$  ( $\lambda_{max}$  460 nm). The present study has as its goal the study of the kinetics of the formation and decay of these products in a shorter time frame than was possible in the earlier conventional xenon flash photolysis.

A detailed investigation of the quantum yields and decay kinetics of hydrated electrons ejected from tryptophan has been carried out. The optical density of the electrons was extrapolated to the instant of laser firing to determine the initial yield. The results were compared to the electron

Figure 9. Initial yield of hydrated electrons as a function of 265-nm laser intensity in ferrocyanide ion and tryptophan.

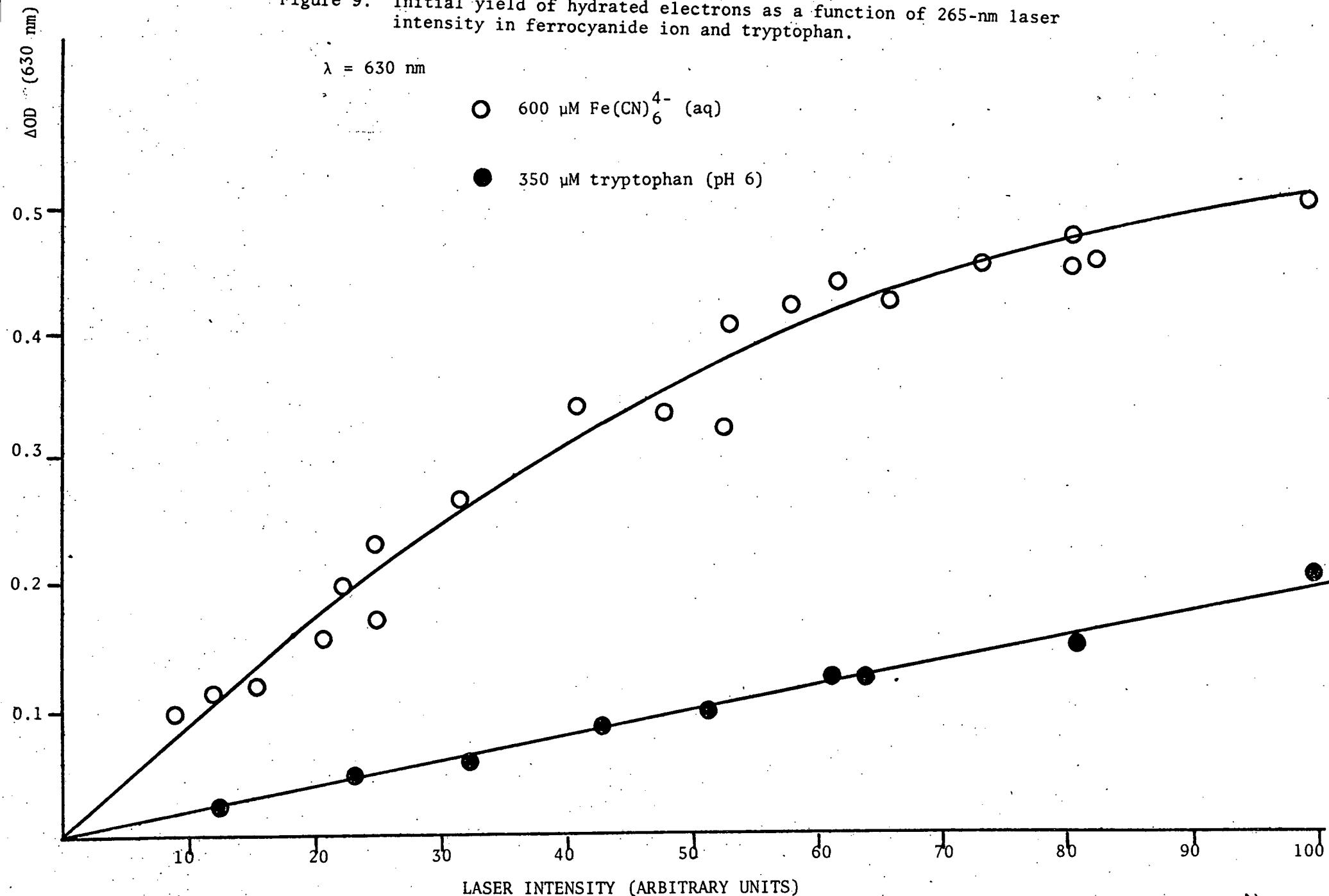


Figure 10. Quantum yield of electron photoejection from 350  $\mu\text{M}$  tryptophan excited at 265 nm,  $\text{N}_2$  saturated.

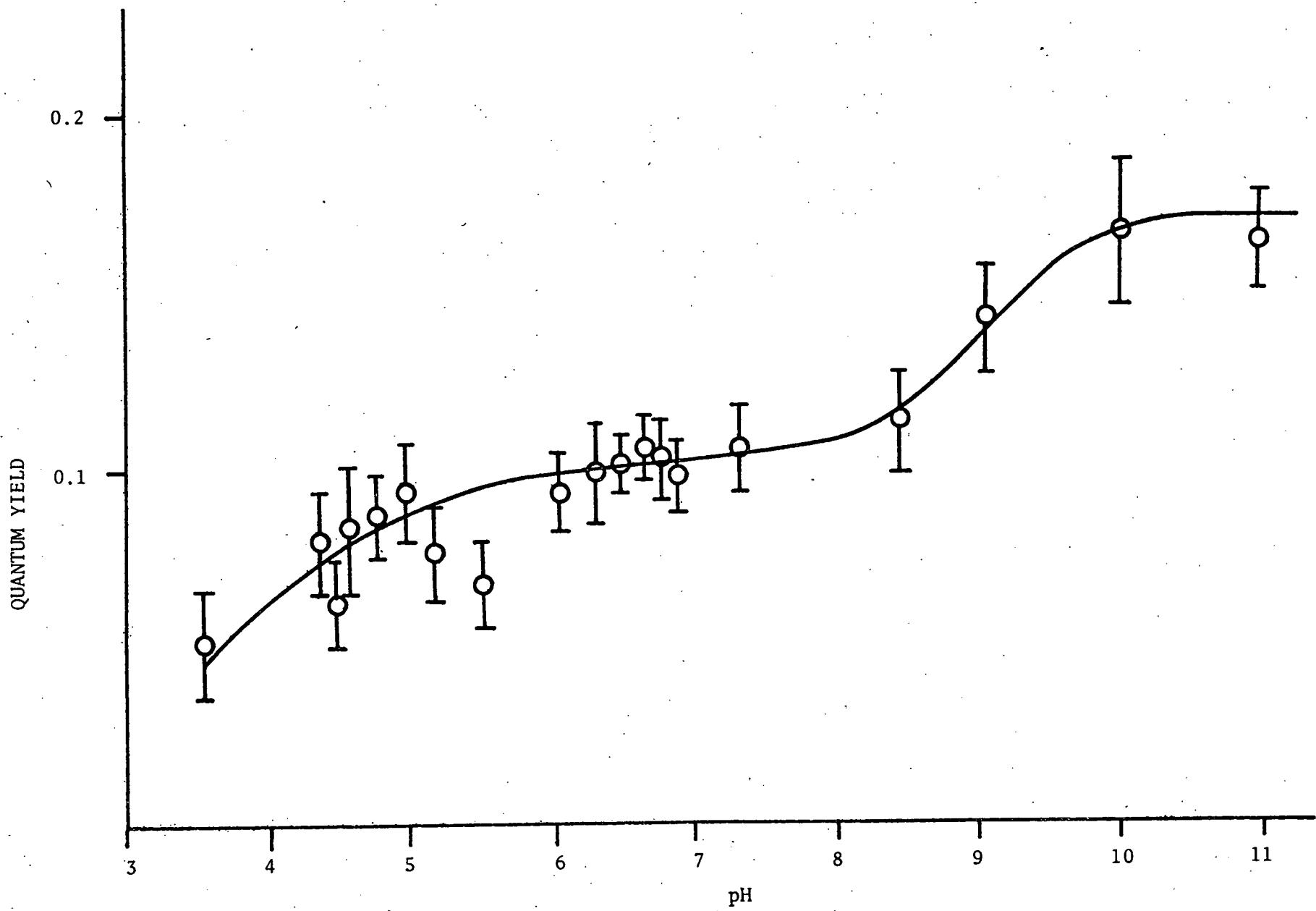


Figure 10a. Lifetime of hydrated electron from 350  $\mu$ M tryptophan after 265-nm laser excitation.

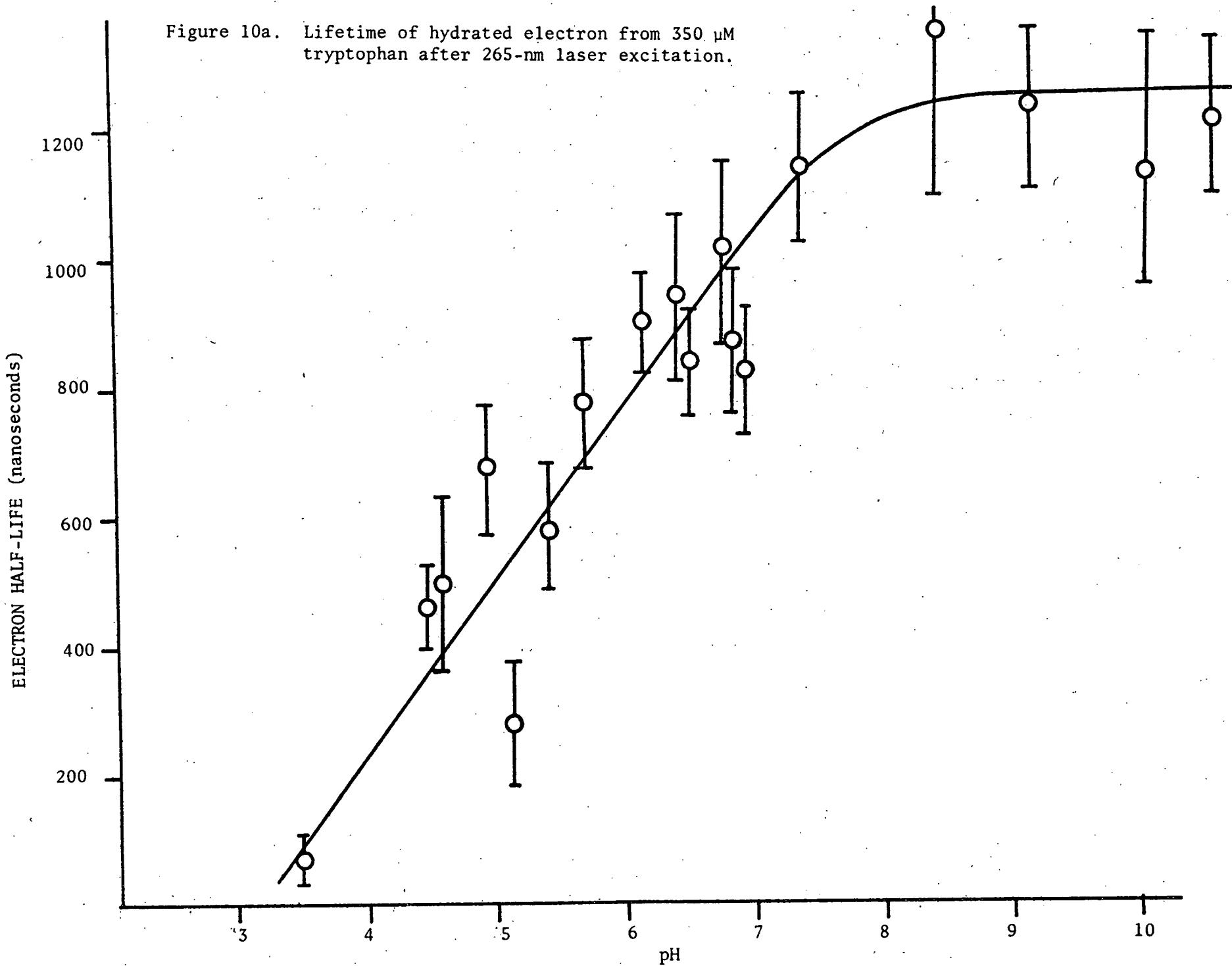
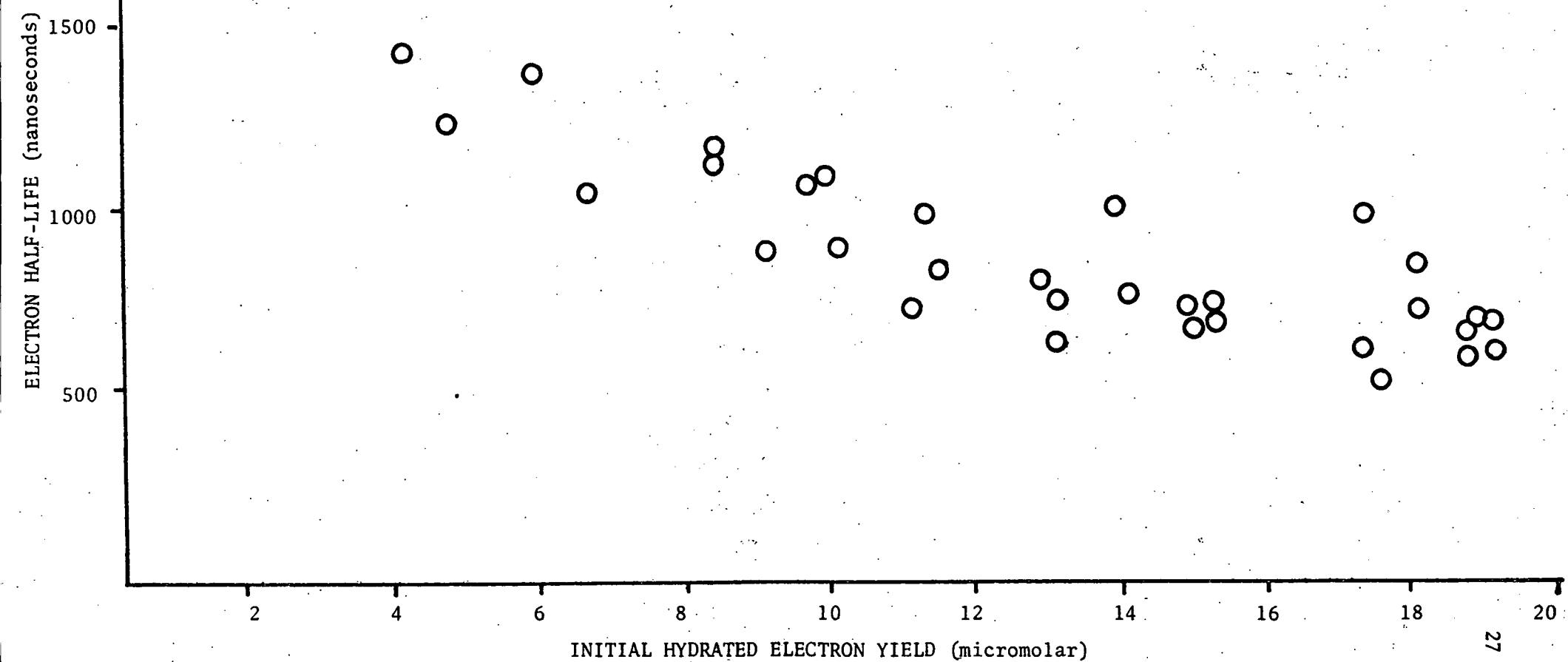


Figure 11. Effect of initial yield on lifetime of photoelectrons produced by 265-nm laser excitation of 350  $\mu$ M tryptophan; pH 6,  $N_2$  saturated.

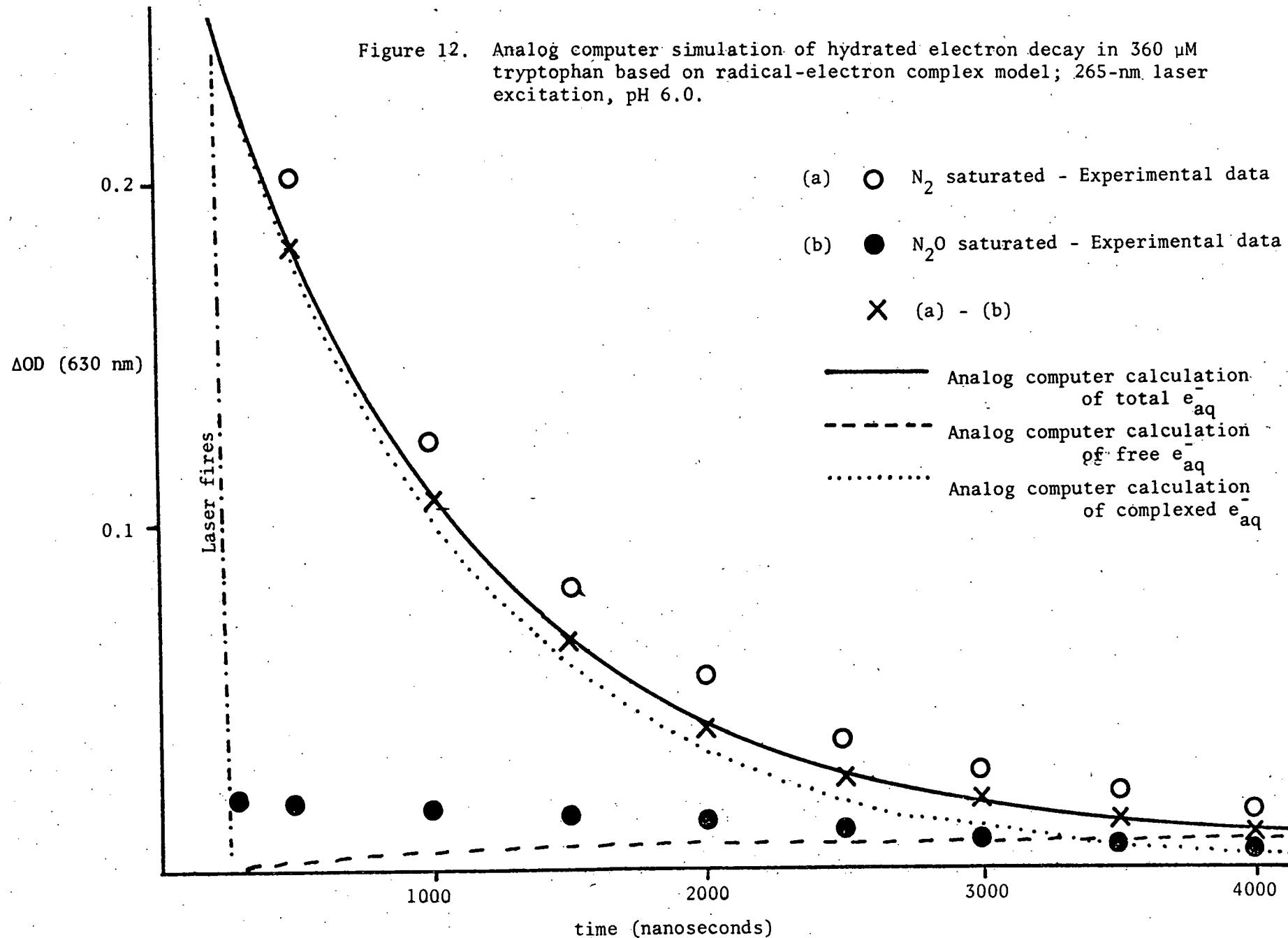


ejection yield from  $K_4Fe(CN)_6$ , based on the ferrocyanide photoionization quantum yield of 0.52 (Shirom and Stein, 1971). A study of the laser intensity dependence of the initial electron yields was made for both tryptophan and ferrocyanide. It was found that the initial photoelectrons yield was linear with laser intensity for tryptophan but not for ferrocyanide (Figure 9). Since the reported photoionization quantum yield of 0.52 was measured at low light intensities, quantum yield measurements in the present case were taken at low laser intensities where the ferrocyanide curve is linear. The quantum yield for electron ejection from tryptophan at natural pH is  $0.10 \pm 0.01$ , in fairly close agreement with the value of 0.08 obtained by Bent and Hayon (1975) using the anthracene T-T absorption as the standard.

The electron ejection quantum yield in tryptophan was measured as a function of pH (Figure 10). The quantum yield is fairly constant at 0.10 between pH 5 and 8. There is a marked increase of quantum yield above pH 8, a value of 0.17 being reached at high pH values. This increase corresponds to the dissociation of the amino group in tryptophan (pK 9.3). There is a decrease in quantum yield below pH 4, apparently a reflection of the protonation of the carboxyl group. It is difficult to measure the hydrated electron decay accurately at these low values of pH because of the rapid reaction of  $e^-_{aq}$  with protons.

The decay rate of  $e^-_{aq}$  is much faster than can be explained by the dismutation reaction or by reaction of electrons with ground state tryptophan. The half-life of the rapid initial electron decay is shown in Figure 10a as a function of pH. The half-life increases monotonically with pH up to pH 9, above which a constant lifetime was observed. The saturation in the lifetime may correspond to the ionization of the amino group of Trp. The half-life of electron decay was also measured as a function of laser intensity (Figure 11).

Figure 12. Analog computer simulation of hydrated electron decay in 360  $\mu\text{M}$  tryptophan based on radical-electron complex model; 265-nm laser excitation, pH 6.0.



<u>Peptide (a)</u>	<u>Electron Quantum Yield</u>	<u>Electron Half Life (nsec)</u>
Trp-Gly	0.077	670
Gly-Trp	0.055	900
Gly-Tyr	0.046	450
Tyr-Gly	0.043	520
Gly-Tyr-Gly	0.031	380

(a) Natural pH

Table V. Electron Ejection from Small Peptides.

Surprisingly, the lifetime is not constant, and decreases monotonically with increasing initial electron yield. The lifetime depends only on the initial electron yield and not on the amount of ground state tryptophan present. The results indicate that a higher order process is involved, despite the good exponential time dependence of the decay.

The electron ejection quantum yields were measured in several small tryptophan and tyrosine-containing peptides. The results are listed in Table V. The quantum yield for electron ejection from tyrosine (at natural pH) was found to be 0.072 and the half-life was 500 nsec.

A detailed analysis of the decay kinetics of the transient species in tryptophan ( $e^-_{aq}$ , Trp,  $^3\text{Trp}$ ) was carried out by simulating the observed data by an analog computer. Laser flash photolysis transient were measured at 630, 520, 460, and 420 nm using oxygen, air, nitrous oxide, and nitrogen as saturated gases. The main difficulty in interpreting such decay patterns is the large amount of overlap between the various species present. Various quenchers were employed to help resolve these spectra. For example, oxygen removes both  $^3\text{Trp}$  and  $e^-_{aq}$ , leaving Trp; nitrous oxide removes only  $e^-_{aq}$ , leaving Trp and  $^3\text{Trp}$ . Furthermore, the species generally decay at different rates, so that the kinetics of two or more species can be isolated by simulating different time regimes of the observed decay.

The radical decay rates and initial yields were evaluated with the analog computer by assuming the photolysis model of Bryant et al. (1975). This model postulates that a loose complex between Trp and  $e^-_{aq}$  is formed immediately following the laser flash. This complex can either recombine to form the ground state of Trp, or else the electron can escape from the radical and equilibrate in the bulk solvent. The decay of the hydrated electron in Trp is shown in Figure 12 with the analog computer simulation. A weakly

Monitoring Wavelength	Saturating Gas	Trp Radical		Triplet State		Hydrated Electron	
		Decay Rate	Initial OD <sup>(b)</sup>	Decay Rate	Initial OD <sup>(c)</sup>	Decay Rate	Initial OD <sup>(d)</sup>
520	oxygen	$1.7 \times 10^5$	0.032	very fast		very fast	
460	oxygen	$1.7 \times 10^5$	0.016	very fast		very fast	
420	oxygen	$1.7 \times 10^5$	0.006	very fast		very fast	
630	air	-	-	-		$4.5 \times 10^6$	0.150
520	air	$1.7 \times 10^5$	0.026	$2.3 \times 10^6$	0.008	$4.5 \times 10^6$	0.075
460	air	$1.7 \times 10^5$	0.010	$2.3 \times 10^6$	0.036	$4.5 \times 10^6$	0.0038
420	air	$1.7 \times 10^5$	0.006	$2.3 \times 10^6$	0.030	$4.5 \times 10^6$	0.002
520	nitrous oxide	$1.7 \times 10^5$	0.030	$8 \times 10^4$	0.008	-	-
460	nitrous oxide	$1.7 \times 10^5$	0.016	$8 \times 10^4$	0.036	-	-
420	nitrous oxide	$1.7 \times 10^5$	0.006	$8 \times 10^4$	0.030	-	-
630	nitrogen	-	-	-	-	$1 \times 10^6$	0.220
520	nitrogen	$1.0 \times 10^6$	0.028	$8 \times 10^4$	0.008	$1 \times 10^6$	0.110
460	nitrogen	$1.0 \times 10^6$	0.016	$8 \times 10^4$	0.036	$1 \times 10^6$	0.055
420	nitrogen	$1.0 \times 10^6$	0.006	$8 \times 10^4$	0.030	$1 \times 10^6$	0.030

(a) natural pH

(e) rate of electron release

(b) 520 nm

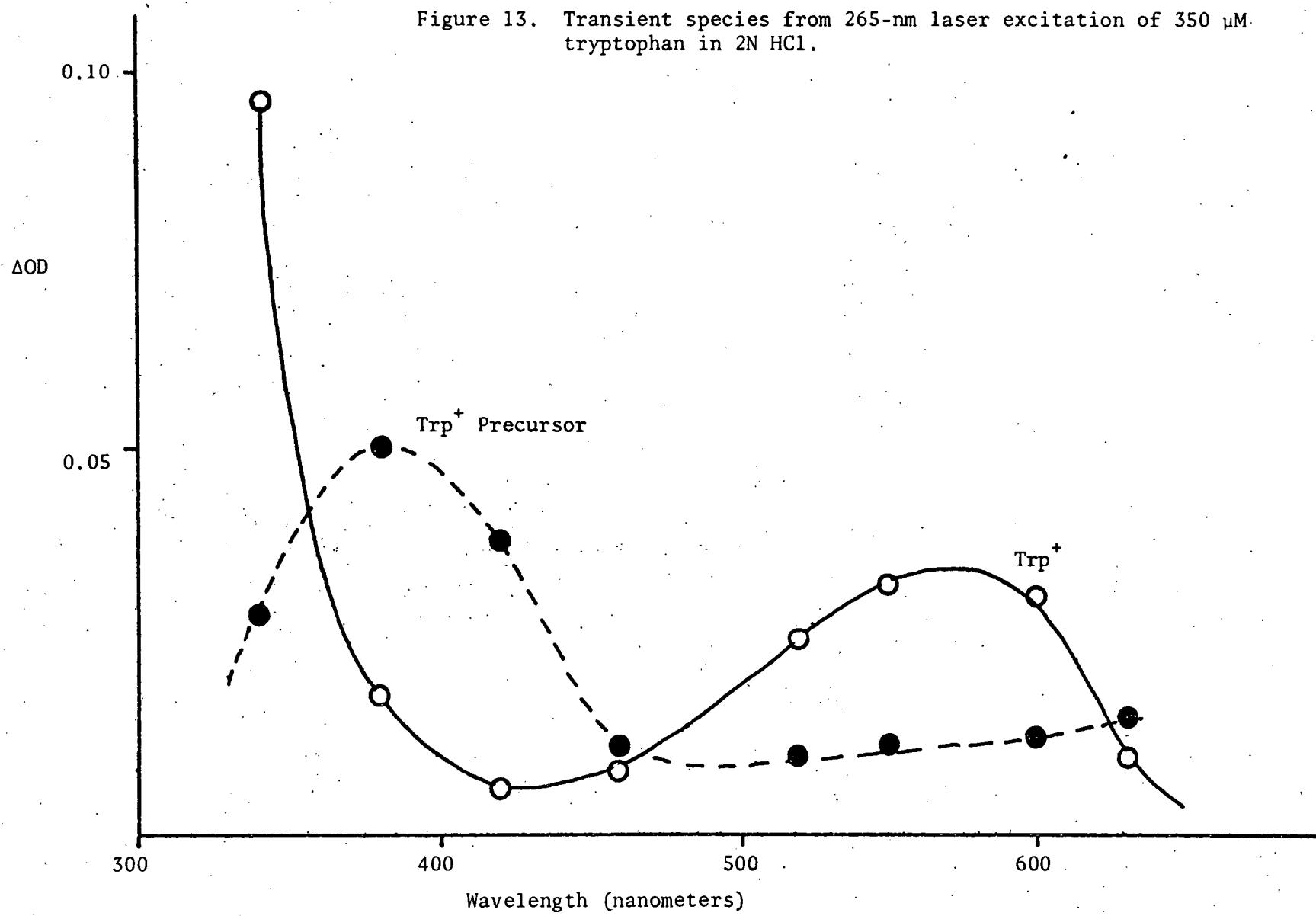
(f) rate of back reaction

(c) 460 nm

(d) 630 nm

Table VI. Analog computer analysis of tryptophan photoionization kinetics.

Figure 13. Transient species from 265-nm laser excitation of 350  $\mu$ M tryptophan in 2N HCl.



absorbing species was present when the sample was saturated with  $N_2O$  which must be subtracted from the nitrogen-saturated decay pattern in order to obtain the electron decay. The results indicate that rapid disappearance of the electron takes place in the complex. At longer times, the decay departs from purely exponential because of the temporary accumulation of electrons that leave the complex and enter the solvent. The analysis indicates that the rate of the back reaction is  $1 \times 10^6 \text{ sec}^{-1}$ , whereas the rate at which the electrons leave the complex is  $5.5 \times 10^4 \text{ sec}^{-1}$ .

The results of the analog computer simulation are summarized in Table VI. The neutral radical Trp decays slowly, and does not react with either oxygen or nitrous oxide. The rapid decay of Trp in  $N_2$ -saturated solutions is a consequence of the rapid recombination of Trp and  $e^-_{aq}$  that takes place when no electron scavengers are present as postulated by the model of Bryant et al. (1975). The presence of electron scavengers tends to interfere with this back reaction, increasing the radical yield at longer times. The triplet state is quenched rapidly by oxygen, at a rate of  $8 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ .

The flash photolysis of tryptophan in  $2N \text{ HCl}$  was studied to determine the role played by protonation in the photoionization process. Transient spectra were taken between 340 to 650 nm at various time delays. Saturation with air or nitrous oxide does not alter the observed results. A species absorbing near 380 nm grows rapidly and decays away. Concurrently, a species absorbing near 580 nm grows in the same rate that the 380 nm species decays. The spectra of these two species are shown in Figure 13. The 580 nm species has been identified as the tryptophan radical cation ( $\text{Trp}^+$ ) by Santus and Grossweiner (1972), and was observed in 1-methyltryptophan at natural pH as well as in Trp at acidic pH. Bryant et al. (1975) measured the molar absorbance of the 1-MeTrp radical cation as  $2600 \text{ M}^{-1} \text{ cm}^{-1}$  at 580 nm. Assuming that the molar absorbance

Monitoring Wavelength	Saturating Gas <sup>(a)</sup>	Trp Radical		Triplet State		RSSR Radical		Hydrated Electron	
		Decay Rate	Initial OD <sup>(b)</sup>	Decay Rate	Initial OD <sup>(c)</sup>	Decay Rate	Initial OD <sup>(d)</sup>	Decay Rate	Initial OD <sup>(e)</sup>
630	nitrogen	-	-	-	-	-	-	$5.7 \times 10^6$	0.200
520	nitrogen	$1.7 \times 10^5$	0.028	$3.5 \times 10^6$	0.008	$7 \times 10^5$	0.015	$5.7 \times 10^6$	0.100
460	nitrogen	$1.7 \times 10^5$	0.016	$3.5 \times 10^6$	0.036	$7 \times 10^5$	0.041	$5.7 \times 10^6$	0.065
420	nitrogen	$1.7 \times 10^5$	0.006	$3.5 \times 10^6$	0.030	$7 \times 10^5$	0.060	$5.7 \times 10^6$	0.040
630	air	-	-	-	-	-	-	$1.0 \times 10^7$	0.180
520	air	$1.7 \times 10^5$	0.027	-	-	$1 \times 10^6$	0.008	$1.0 \times 10^7$	0.090
460	air	$1.7 \times 10^5$	0.016	-	-	$1 \times 10^6$	0.022	$1.0 \times 10^7$	0.045
420	air	$1.7 \times 10^5$	0.006	-	-	$1 \times 10^6$	0.030	$1.0 \times 10^7$	0.031
520	N <sub>2</sub> O	$1.7 \times 10^5$	0.031	$3.5 \times 10^6$	0.008	$5 \times 10^5$	0.006	-	-
460	N <sub>2</sub> O	$1.7 \times 10^5$	0.018	$3.5 \times 10^6$	0.036	$5 \times 10^5$	0.020	-	-
420	N <sub>2</sub> O	$1.7 \times 10^5$	0.006	$3.5 \times 10^6$	0.030	$5 \times 10^5$	0.028	-	-
520	O <sub>2</sub>	$1.7 \times 10^5$	0.032	-	-	$1 \times 10^6$	0.002	-	-
460	O <sub>2</sub>	$1.7 \times 10^5$	0.016	-	-	$1 \times 10^6$	0.008	-	-
420	O <sub>2</sub>	$1.7 \times 10^5$	0.006	-	-	$1 \times 10^6$	0.011	-	-

(a) 360  $\mu\text{M}$  tryptophan + 465  $\mu\text{M}$  cystine, natural pH.

(b) 520 nm

(c) 460 nm

(d) 420 nm

(e) 630 nm

Table VII. Analog computer analysis of tryptophan photoionization in the presence of cystine.

of  $\text{Trp}^+$  is the same as this species, the quantum yield for  $\text{Trp}^+$  formation is about 0.068 in 2N HCl. The transient absorbing at 380 nm is apparently the precursor of  $\text{Trp}^+$ , since it decays at the same rate that  $\text{Trp}^+$  grows in. We have no information at present as to the nature of this species.

An important question involves the means by which light energy absorbed by a chromophoric amino acid residues leads to photolysis of disulfide bridges. To gain some insight into this process, a laser flash photolysis study was undertaken on mixtures of tryptophan and cystine.

The observed transient decays were simulated with the analog computer in the same way as in the case of tryptophan and the results are summarized in Table VII. The presence of cystine does not alter the decay of  $\text{Trp}$ , indicating that this radical does not react with RSSR. There is, however, a rapid reaction between  $^3\text{Trp}$  and cystine, the cystine radical anion (RSSR) being formed. There is also a rapid reaction between  $e_{\text{aq}}^-$  and RSSR which also produces RSSR. The decay of RSSR is rapid ( $7 \times 10^5 \text{ sec}^{-1}$ ). The presence of oxygen markedly impedes the formation of RSSR, since  $O_2$  reacts with both  $e_{\text{aq}}^-$  and  $^3\text{Trp}$ . However,  $N_2O$  reacts only with  $e_{\text{aq}}^-$ , leaving the reaction between  $^3\text{Trp}$  and cystine unaffected. If it is assumed that all of the  $^3\text{Trp}$  reacting with cystine under  $N_2O$  saturation produce RSSR, the extinction coefficient of  $^3\text{Trp}$  can be determined. The results are  $\epsilon(^3\text{Trp}) = 11,000 \text{ M}^{-1} \text{ cm}^{-1}$  (460 nm),  $9000 \text{ M}^{-1} \text{ cm}^{-1}$  (420 nm), and  $2500 \text{ M}^{-1} \text{ cm}^{-1}$  (520 nm). The quantum yield for  $^3\text{Trp}$  formation is 0.02.

<u>Enzyme</u>	<u>Concentration (μM)</u>	<u>pH</u>	<u>Electron Quantum Yield</u>	<u>Decay Half-life (nsec)</u>
papain	47	7.0	0.042	560
Carbonic anhydrase	50	5.0	0.024	290
Lysozyme (hen)	56	5.5	0.013	210
Trypsin (bovine)	65	3.8	0.025	220
Ribonuclease A (bovine)	240	3.8	≤ 0.006	-
	240	5.8	0.013	300
	240	11.6 (KOH)	0.063	1250
Subtilisin Carlsberg	115	6.3	0.044	400
Subtilisin Novo	77	6.4	0.042	370
Subtilisin BPN'	80	5.8	0.046	360
Catalase (beef liver)		7.1	0.059	700

Table VIII. Initial electron yields and electron decay lifetimes in 265 nm laser photolysis of enzymes.

### 3. Laser Flash Photolysis of Proteins

Laser flash photolysis studies of enzymes have been initiated with the objectives of identifying the intermediate species present within 20 nsec of irradiation and studying their subsequent fates. The initial effort has been on studying the hydrated electrons ejected from the aromatic residues in proteins. A number of proteins were examined to gain some preliminary information about the initial electron yields and lifetimes. The results are given in Table VIII. The electron quantum yields range between 0.01 and 0.05 at 265 nm and the electron lifetimes vary from 200 and 700 nanoseconds.

A detailed examination of the electron decay kinetics in papain was made, so that a comparison could be made between the electrons ejected from aromatic amino acids in solution with electron ejected from proteins. In papain, the decay of the electron appears to be purely exponential and the rate of decay is independent of laser intensity. However, the decay rate is linearly proportional to the papain concentration, indicating a second-order reaction between  $e^-_{aq}$  and papain. The apparent second-order rate constant for papain is  $1.4 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ , compared with the value of  $4.1 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$  reported for pulse-radiolysis of papain solutions (Clement, 1974). The rate of oxygen reaction with electrons ejected from papain is  $1 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ , appreciably smaller than the value of  $2 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$  determined by pulse radiolysis. These results suggest that the electron generated by the photoionization of aromatic residues in papain is less accessible to external electron quenchers than the hydrated electron produced in water radiolysis.

A detailed study of the decay kinetics of other species formed in the laser photolysis of papain is underway, in order to compare the yields and decay times with those in aqueous tryptophan. In oxygen-saturated solutions of papain, the initial quantum yields of Trp and RSSR are 0.044 and 0.009

respectively. The decay of Trp was very slow, whereas the decay rate of RSSR was  $\sim 2 \times 10^5$  sec $^{-1}$ . RSSR appeared within the time resolution of the laser experiment ( $\sim 20$  nsec), and appears to be formed by a rapid internal transfer from Trp to cystine. However, only about 20 percent of the electrons ejected from Trp are located at cystine bridges at short times after the flash. The other ejected electrons are either transferred elsewhere within the molecule or are scavenged by external quenchers such as oxygen or other papain molecules. Further studies are under way on papain in nitrogen and nitrous oxide-saturated samples, in which the triplet state of tryptophan makes an appreciable contribution to the flash photolysis spectrum.

The fluorescence yield of Trp in trypsin is markedly enhanced when 8M urea is present, reflecting the extensive unfolding of the molecule making tryptophan molecules buried in the interior more accessible to the solvent (Burstein et al., 1973). A study of the laser flash photolysis of denatured trypsin in 8M urea was undertaken to determine the effect on the electron photoejection quantum yield. It was found that the electron quantum yield for tryptophan in 8M urea was 0.12, only slightly higher than in aqueous solution. However, the quantum yield for electron photoejection in trypsin was nearly doubled when 8M urea was added. The enhanced quantum yield in 8M urea parallels the increased fluorescence yield, indicating that photoionization of tryptophan in proteins is closely related to solvent accessibility.

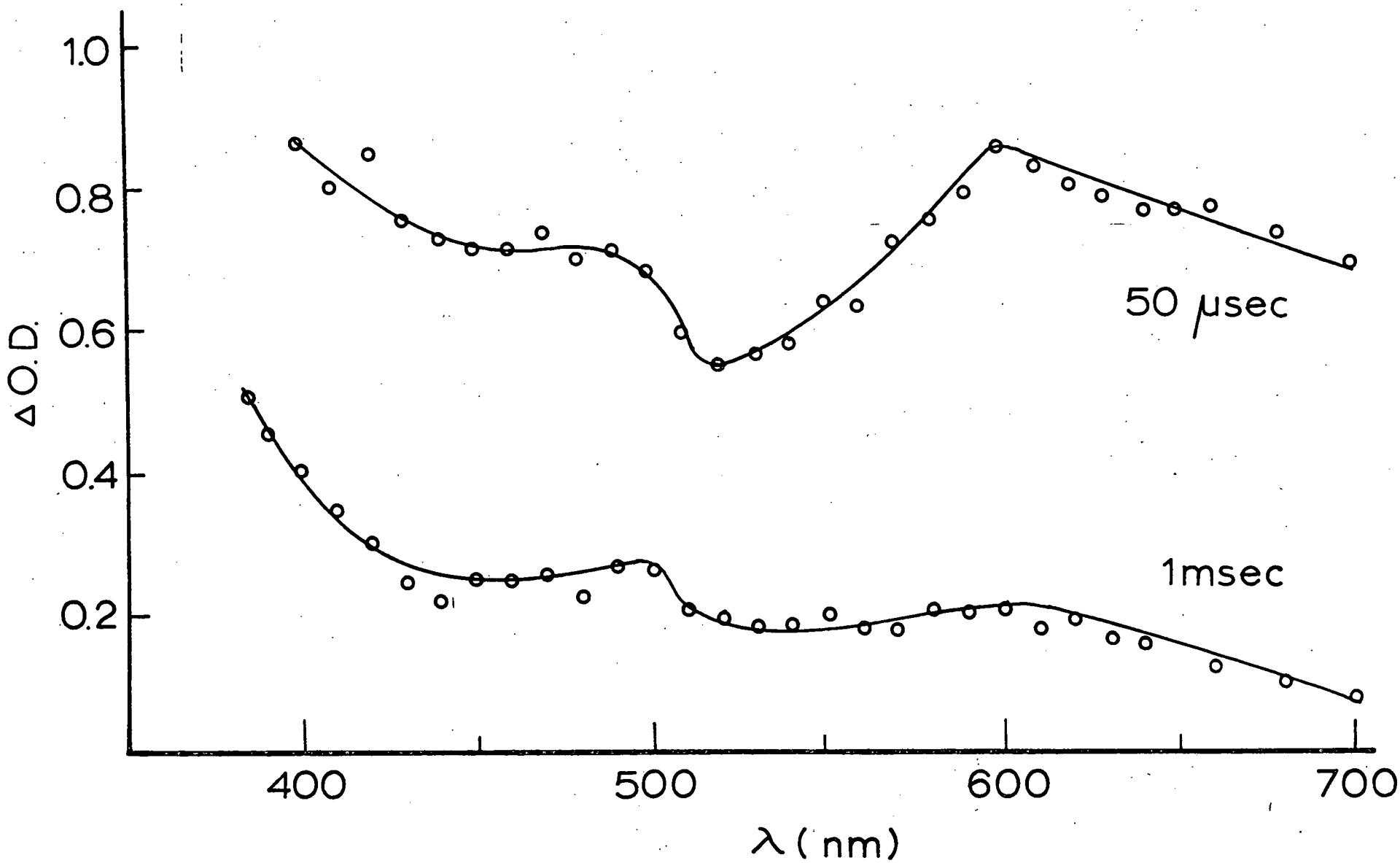


Figure 14. Flash photolysis spectra from 74  $\mu$ M 8-Methoxysoralen in glycerole, nitrogen-saturated,  $\lambda > 305$  nm.

4 . Photodynamic Sensitization by 8-Methoxypsoralen via the Singlet Oxygen Mechanism

There is ample evidence that biological sensitization by furocoumarins does not require oxygen (e.g. Musajo and Rhodighiero, 1972). It is not known how furocoumarins differ from the many synthetic dyes and natural pigments that act as photodynamic sensitizers via the type II (dye-oxygen) mechanism. We have found that 8-MOP is a good photodynamic sensitizer in aqueous solution, and that singlet oxygen ( $^1\text{O}_2$ ) generated by triplet energy transfer is the principal reactive intermediate in the inactivation of lysozyme and oxidation of aqueous iodide ion. Evidence that the binding of 8-MOP to double stranded poly(dA-dT) inhibits its ability to sensitize via this mechanism has been obtained.

The triplet state of 8-MOP was identified by flash photolysis in aqueous and glycerol solutions. Figure 14 shows the transient absorption generated by irradiating 74  $\mu\text{M}$  8-MOP in glycerol through a 0.1% potassium hydrogen phthalate filter ( $\lambda > 305$  nm). The decay can be analyzed into two, first-order stages of 200  $\mu\text{s}$  and  $\sim$ 35 ms lifetimes where the longer-lived species is attributed to a secondary product. Similar transient bands at 600, 480 and  $<360$  nm were obtained in water but the lifetime was considerably shorter. The quadrupled Nd-glass laser providing 17 ns pulses at 265 nm was employed to measure the decay lifetimes of 1.8  $\mu\text{s}$  in deaerated solutions and 0.4  $\mu\text{s}$  under oxygen saturation. The decreased decay lifetime in oxygen corresponds to a quenching rate constant of approximately  $1 \times 10^9 \text{ l/mol-s}$ , typical of triplet states.

The possibility that the transient absorption is a relatively long-lived, excited singlet state was eliminated by measuring the fluorescence lifetime. Air-saturated solutions of 65  $\mu\text{M}$  8-MOP were excited with a repetitive, 3 ns flash lamp (Photochemical Research Associates Model 510)

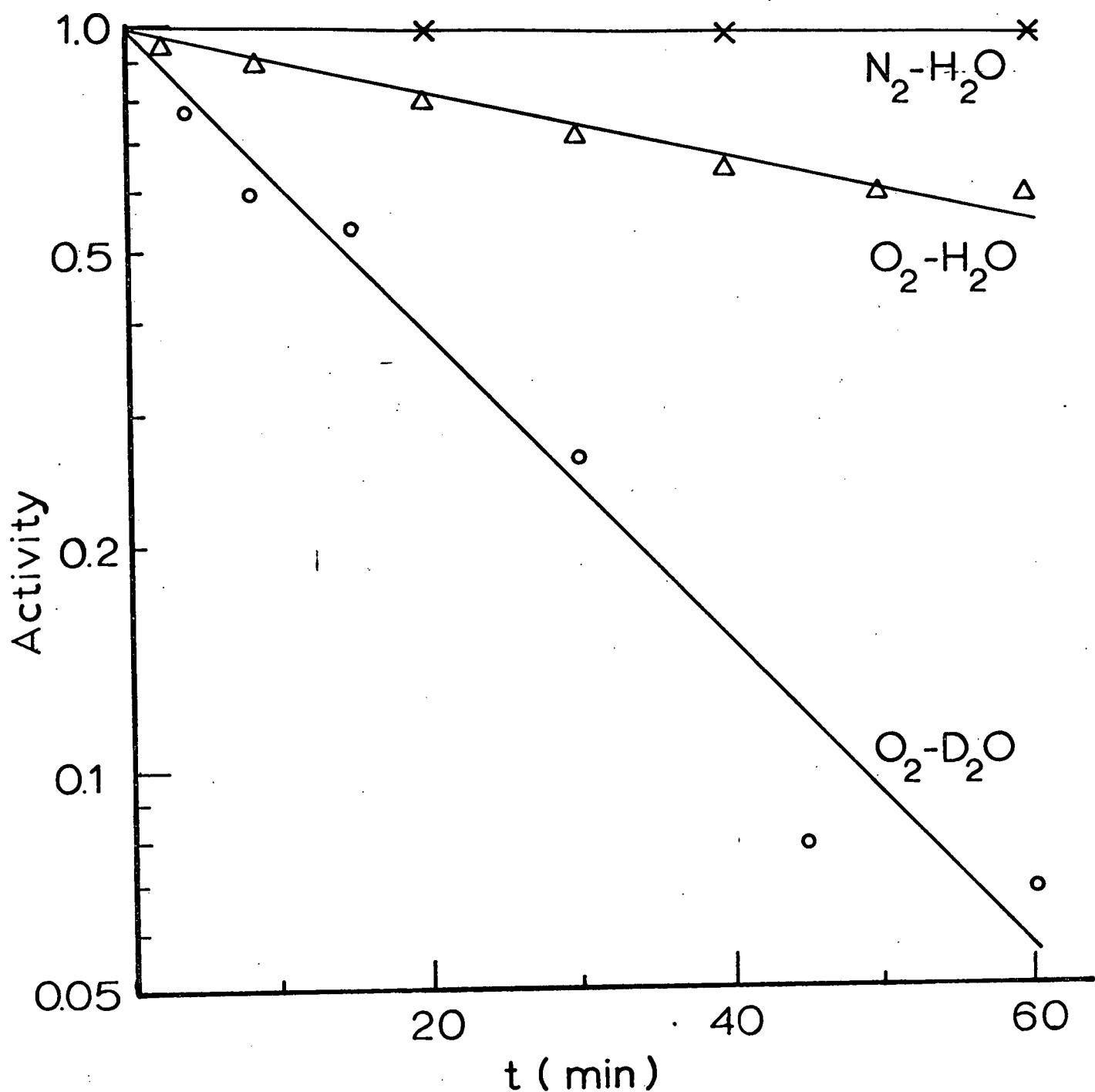


Figure 15. Photodynamic inactivation of 20  $\mu\text{M}$  lysozyme sensitized by 29  $\mu\text{M}$  8-Methoxysoralen ;  $\lambda > 305 \text{ nm}$ , no buffer.

through a filter transmitting from 240 nm to 380 nm and the luminescence pulses were detected with a 1P28 photomultiplier and integrated with a PAR Model 162 Boxcar Averager. Fluorescence decay lifetimes of 1.9 ns in water and 2.5 ns in glycerol were calculated using a 'least squares' deconvolution analysis on a PDP-11/45 Computer.

The ability of 8-MOP to act as a photodynamic sensitizer of lysozyme inactivation is shown in Figure 15. The solutions were irradiated with a 200-W h.p. Hg-Xe arc at 18°C ( $\lambda > 305$  nm) and the resultant enzymic activity assayed with Micrococcus lysodeikticus. The enhanced rate of inactivation in  $D_2O$  solvent compared with  $H_2O$  was observed previously with acridine orange (Schmidt and Rosenkranz, 1972) and eosin (Kepka and Grossweiner, 1973) as sensitizers and attributed to the corresponding increase in the  $^1O_2$  decay lifetime from 2  $\mu$ s to 20  $\mu$ s (Merkel et al., 1972). The dependence of the relative inactivation rate on lysozyme concentration was analyzed by assuming that scavenging of  $^1O_2$  competes with the solvent-induced decay according to

$$\phi_{in} = \phi_{\Delta} \frac{k'_L [L]}{1/\tau + k_L [L]} \quad (1)$$

where  $\phi_{in}$  and  $\phi_{\Delta}$  are the inactivation and  $^1O_2$  production quantum yields, respectively,  $k'_L$  and  $k_L$  are the inactivation and total quenching rate constants, respectively, and  $\tau$  is the  $^1O_2$  decay lifetime. The 'double reciprocal' plot of (1) for a series of runs with oxygen-saturated, 20  $\mu$ M 8-MOP solutions in  $D_2O$  leads to  $k_L \tau = 2.5 \times 10^4$   $\text{M}^{-1} \text{s}$  and  $k_L = 1.3 \times 10^9$   $\text{M}^{-1} \text{s}$ . This result is in satisfactory agreement with  $k_L = 1.9 \times 10^9$  reported by Kepka and Grossweiner (1973) for lysozyme sensitization by eosin and  $1.5 \times 10^9$  obtained by Matheson et al. (1975) where  $^1O_2$  was generated directly by a Nd-YAG laser. The ' $D_2O$  test' was employed also to show that

<u>Sensitizer</u>	<u>Substrate</u>	<u>Conditions</u>	<u>p(dA-dT)</u>	<u>Relative Sensitivity</u>
8-methoxypsonalen (29 $\mu$ M)	20 $\mu$ M Lysozyme	N <sub>2</sub> , H <sub>2</sub> O	-	~0.0 (a)
"	"	O <sub>2</sub> , H <sub>2</sub> O	-	1.0
"	"	O <sub>2</sub> , D <sub>2</sub> O	-	4.6
8-methoxypsonalen (5 $\mu$ M)	0.1M I <sup>-</sup>	N <sub>2</sub> , D <sub>2</sub> O	-	~0.0 (b)
"	"	Air, H <sub>2</sub> O	-	1.0
"	"	Air, D <sub>2</sub> O	-	3.7
"	"	Air, D <sub>2</sub> O	30 $\mu$ M (c)	2.5
"	"	Air, D <sub>2</sub> O	75 $\mu$ M (c)	1.7
Eosin (3 $\mu$ M)	0.1M I <sup>-</sup>	N <sub>2</sub> , D <sub>2</sub> O	-	~0.0
"	"	Air, H <sub>2</sub> O	-	1.0
"	"	Air, H <sub>2</sub> O	30 $\mu$ M (c)	0.92
"	"	Air, D <sub>2</sub> O	-	1.79
"	"	Air, D <sub>2</sub> O	30 $\mu$ M (c)	1.68

(a) Lysozyme assayed with Micrococcus lysodeikticus

(b) I<sub>3</sub><sup>-</sup> formation measured at 352 nm and corrected for 8-MOP

(c) thymine equivalent

(d) The quantum yield for photosensitized inactivation of 20  $\mu$ M lysozyme in the presence of 20  $\mu$ M 8-MOP (pH ~7) is 0.0036 in air-sat H<sub>2</sub>O and 0.0165 in air-sat D<sub>2</sub>O.

Table IX. Photodynamic activity of 8-methoxypsonalen.

8-MOP sensitizes the photooxidation of  $I^-$  to  $I_3^-$  (Table IX). Previous work of Kepka and Grossweiner (1973) demonstrated that  $^1O_2$  is the major oxidizing agent of  $I^-$  with eosin sensitization, in further support of the type II photodynamic activity of 8-MOP.

The effect of binding on the sensitizing ability of 8-MOP was shown by adding double-stranded poly(dA-dT) to the sensitizer-substrate system. [This test was limited to  $I^-$  photooxidation because of complexing between lysozyme and poly(dA-dT)]. The results in Table IX show that poly(dA-dT) does not alter significantly the ability of eosin to sensitize  $I^-$  oxidation in  $H_2O$  or  $D_2O$  solutions. However, the presence of 75  $\mu M$  thymine equivalent poly(dA-dT) diminishes the sensitizing efficiency of 5  $\mu M$  8-MOP by more than a factor of 2 in  $D_2O$ . The negative result with eosin rules out the possibility of  $^1O_2$  quenching by poly(dA-dT) under the equivalent reaction conditions, suggesting that 8-MOP binding to poly(dA-dT) inhibits the type II pathway.

It does not necessarily follow that the competing type I (dye-substrate) pathway involves the triplet state. Bevilacqua and Bordin (1973) concluded that the triplet state of psoralen reacts with free pyrimidine bases from the quenching effects of oxygen and paramagnetic salts on the rate of photo-adduct formation. However, dark binding probably was much weaker compared to the polynucleotides and, in fact, the extent of paramagnetic quenching diminished at high base-to-psoralen molar ratios indicative of excited-singlet sensitization. The present results suggest that additional studies should be made on the molecular structures and irradiation conditions that suppress  $^1O_2$  formation in connection with clinical applications of furocoumarins, in view of the high potential for cell damage by this diffusive reactive intermediate.

##### 5. Photodynamic and Photosensitized Inactivation of Yeast

The biological effects of photodynamic action are being investigated in yeast cells with emphasis on damage to cell membranes in vivo. Yeast provides a particularly advantageous system for these studies since the organism multiplies rapidly and can be cultured on relatively simple media. The osmotic stability provided by the cell wall allows yeast to be suspended in water rather than a nutrient medium for irradiation, so that no inadvertent potential sensitizers are present. In addition, yeast is a eukaryotic micro-organism, so that the results may provide the basis for understanding of photoirradiation damage in higher eukaryotes.

###### Eosin-sensitized Photodynamic Inactivation of Yeast

Previous measurements have shown that the dye, eosin Y, sensitizes yeast inactivation without producing toxic effects in the absence of light (see Progress Report 7/1/74 - 6/30/75). In addition, we demonstrated previously that washing yeast with distilled water following incubation in aqueous eosin Y prevents subsequent photodynamic damage, as assayed by survival measurements and post-irradiation doubling-time determinations. These data indicate that the observed inactivation was caused by light only and that the eosin either remains outside the cell or is weakly bound (Tseng and Cohn, 1976).

Figure 16 presents typical survival curves obtained by irradiating strain Y55 diploid yeast with visible light ( $\lambda > 400$ ) in the presence of oxygen and eosin Y. The cells were washed twice with distilled water and were re-suspended in sterile aqueous eosin for irradiation. Parallel measurements for irradiation of nitrogen-saturated yeast suspensions in the presence of eosin show a marked reduction in the degree of cell inactivation. The survival curve parameters are summarized in Table X. These results show that inactivation

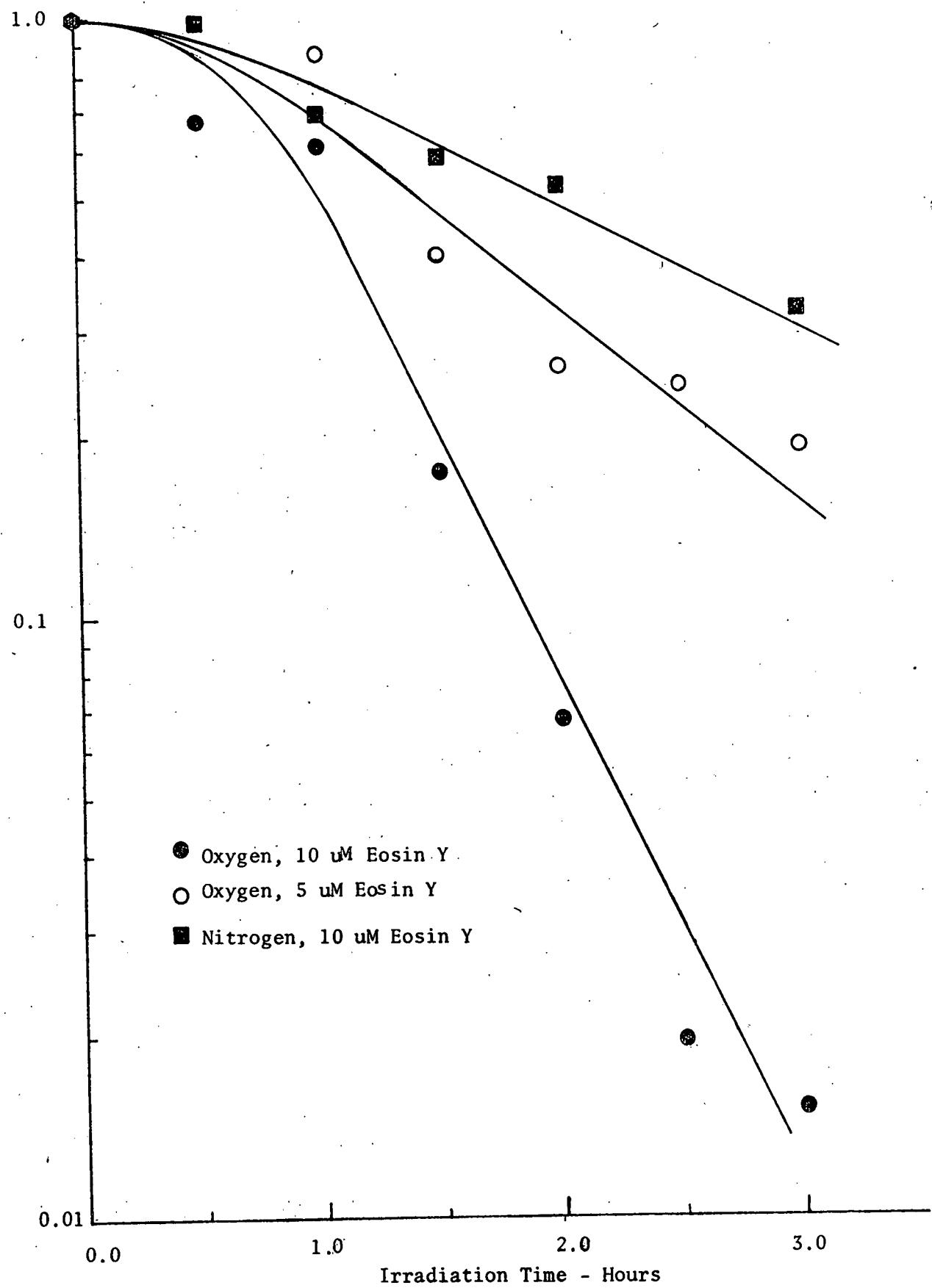


Figure 16. Photodynamic inactivation of diploid yeast sensitized by eosin Y.

Eosin Concentration ( $\mu$ M)	Temperature ( $^{\circ}$ C)	Saturating Gas	$D_0$ (a)	Relative Sensitivity	Extrapolation Number
5	27	$O_2$	$3.45 \times 10^{10}$	0.055	1.0
5	30	$O_2$	$4.53 \times 10^9$	0.422	1.6
10	30	$O_2$	$1.91 \times 10^9$	1.00	3.3
2	30	$N_2$	$7.41 \times 10^9$	0.258	1.1
10	30	$N_2$	$3.56 \times 10^9$	0.537	1.3

(a) linear region:  $\text{erg/cm}^2$  from 400 nm to 550 nm

Table X. Log-survival vs dose parameters for yeast inactivation photosensitized by eosin.

Eosin Concentration ( $\mu$ M)	(a) $D_0$	Relative Sensitivity	Extrapolation Number
5	$1.48 \times 10^9$	0.364	4.2
10	$5.39 \times 10^8$	1.00	162

(a)  
linear region:  $\text{erg/cm}^2$  from 400 nm to 550 nm.

Table XI. Correction of yeast photoinactivation data of Table X  
for photobleaching of eosin.

is caused primarily by eosin-sensitized photodynamic attack, although some degree of anoxic sensitization may be involved. In view of the previous results suggesting that eosin Y is extracellular sensitizer, the plasma membrane must be considered as a possible primary target for the damaging agents generated by eosin Y.

Eosin Y is bleached to a non-absorbing species during light irradiation (Imamura, 1956; Usui et al., 1965) and the consequent reduction of available sensitizer molecules must affect the observed rates of inactivation. Measurements of the rate of eosin bleaching were made for aqueous solutions of the dye in order to correct the observed survival curves. The following expression has been derived for the survival curve which would be obtained in the absence of bleaching (Tseng and Cohn, 1976):

$$S(t) = \exp - \left\{ \frac{k_e \log_e [N_y(t)/mN_0]}{e^{-k_e t} - 1} \right\} \quad (2)$$

where  $k_e$  is the observed dye bleaching rate constant,  $N_y(t)/N_0$  is the measured survival fraction after  $t$  seconds of light exposure,  $m$  is the extrapolation number and  $S(t)$  is the corrected survival fraction. The data of Fig. 16 corrected in this manner are plotted in Fig. 17, and survival curve parameters measured from these curves are listed in Table XI. A comparison of these results with Fig. 16 and Table X shows that the actual photodynamic inactivation of yeast is much more effective than the uncorrected results would indicate and there is a significant shoulder at low doses. These calculations assume only that eosin does not interact with the cells to any significant degree in the dark and that eosin added to yeast absorbs light as it would in distilled water.

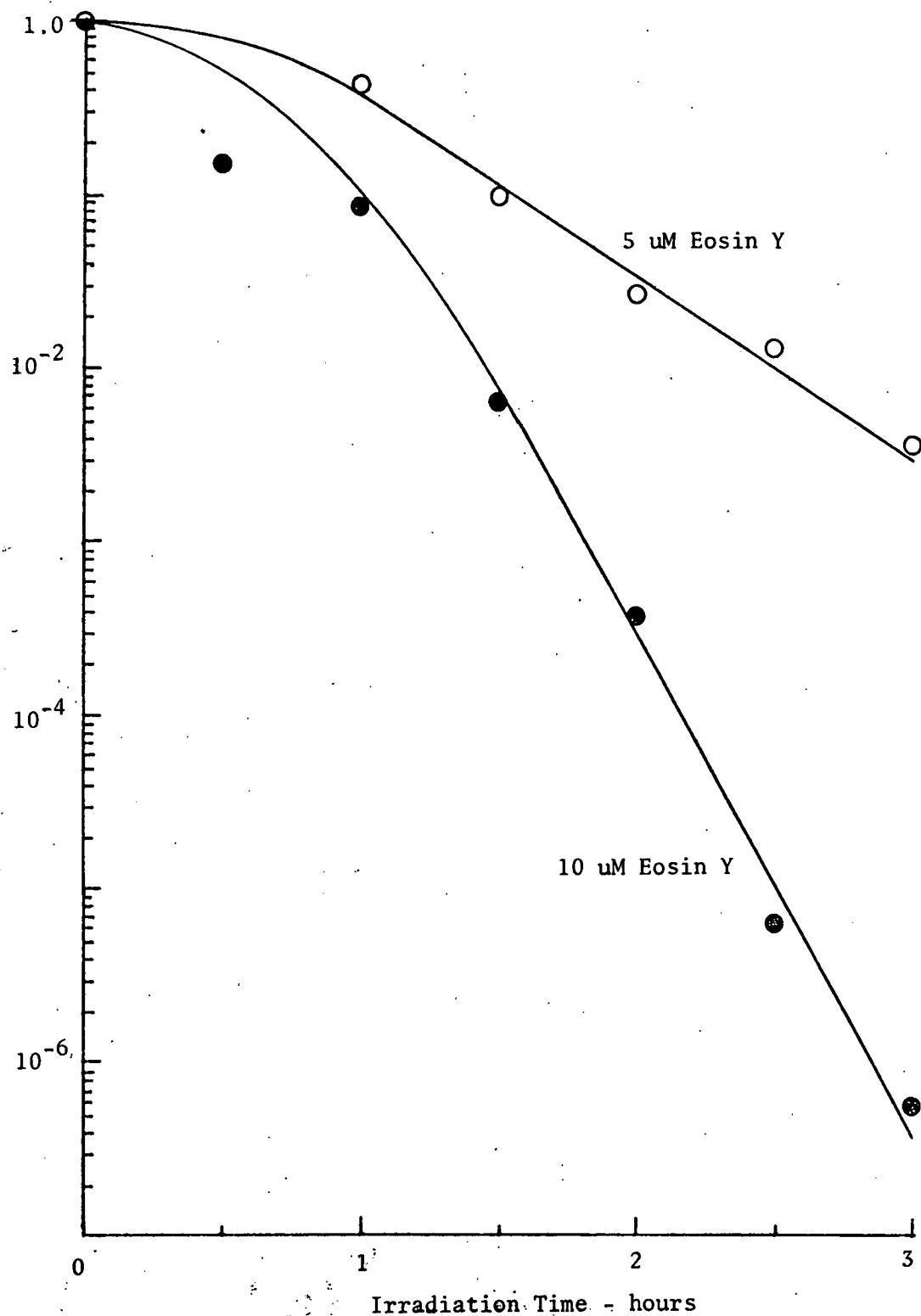


Figure 17. Photodynamic inactivation of diploid yeast sensitized by eosin Y, corrected for dye bleaching.

### Photoinactivation of Yeast Sensitized by 8-Methoxypsoralen

The drug 8-methoxypsoralen (8-MOP) is an anoxic photosensitizer of cell killing which acts by forming photoadducts with DAN (Musajo and Rodighiero, 1972). 8-MOP has been shown to be an active sensitizer for irradiation by UV-A light in the bacteria Sarcina lutea (Mathews, 1963), Staphylococcus aureus and Escherichea coli (Oginsky et al., 1959) and Bacillus subtilis (Song et al., 1975) as well as in mammalian cells (Epstein and Fukuyama, 1975). Motivated by the current interest in 8-MOP for use in the phototherapy of psoriasis, we have undertaken an investigation of the cellulae inactivation processes sensitized by 8-MOP in the Y55 yeast strain.

Yeast were grown to mid-logarythmic phase in standard YNBD 0.5 liquid medium (6.8g Difco Yeast Nitrogen Base + 5g dextrose per liter), washed twice in sterile distilled water and resuspended in a sterile aqueous solution of 8-MOP. After incubation for 20 minutes, the cells were irradiated with UV-A ( $\lambda \geq 310$  mm) in the incubating medium in the presence of either oxygen or nitrogen, and aliquots were withdrawn for serial dilution and plating. The resulting survival curves plotted in Figure 18 demonstrate that 8-MOP is an extremely potent sensitizer of yeast photoinactivation. At higher doses the exponentially decreasing regions of the survival curves were approximately parallel but the shoulder observed at low doses was lengthened in the case of oxygen saturation. No killing was observed indicating that the results of Figure 18 are not caused by an endogenous anoxic photosensitizer. Yeast plated on nutrient agar medium containing up to 60  $\mu$ M 8-MOP showed no reduction in colony-forming ability, indicating that the sensitizer itself is not toxic.

Diluted cell suspensions were held at room temperature in the dark for five days following postirradiation plating in order to assay for possible dark recovery. Since the onset of exponential killing requires larger doses

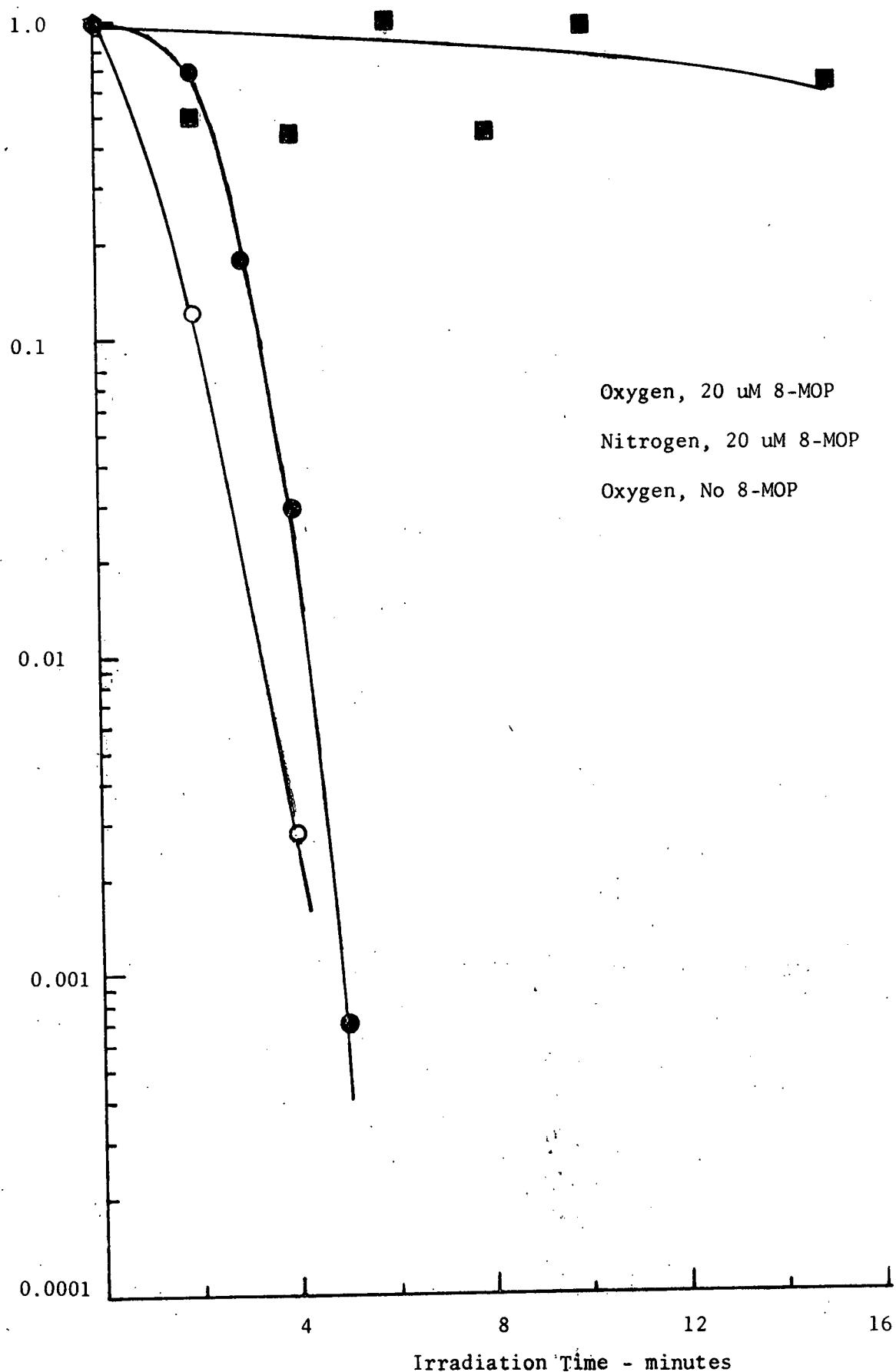


Figure 18. Photodynamic inactivation of diploid yeast sensitized by 8-methoxysoralen.

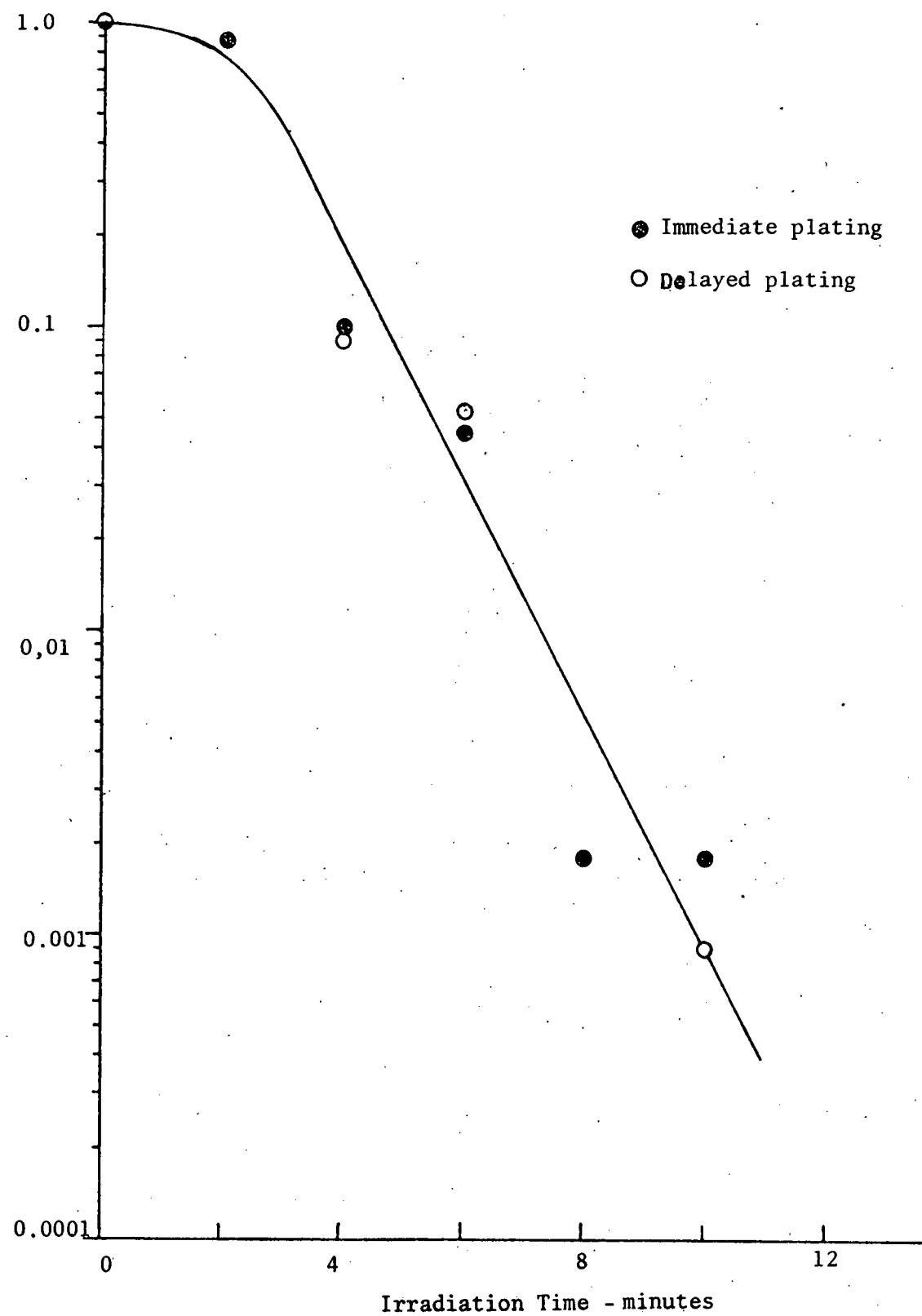


Figure 19. Dark recovery of diploid yeast from 8-MOP-photosensitized UVA irradiation.

under oxygen saturation than under nitrogen saturation, it is possible that repair processes in the former case increase the cells' apparent radiation resistance. The data of Figure 19 show that no significant dark recovery occurs, suggesting that the extended shoulder for irradiation in the presence of oxygen may be from the oxygen-dependent inhibition of the primary photochemical lesion.

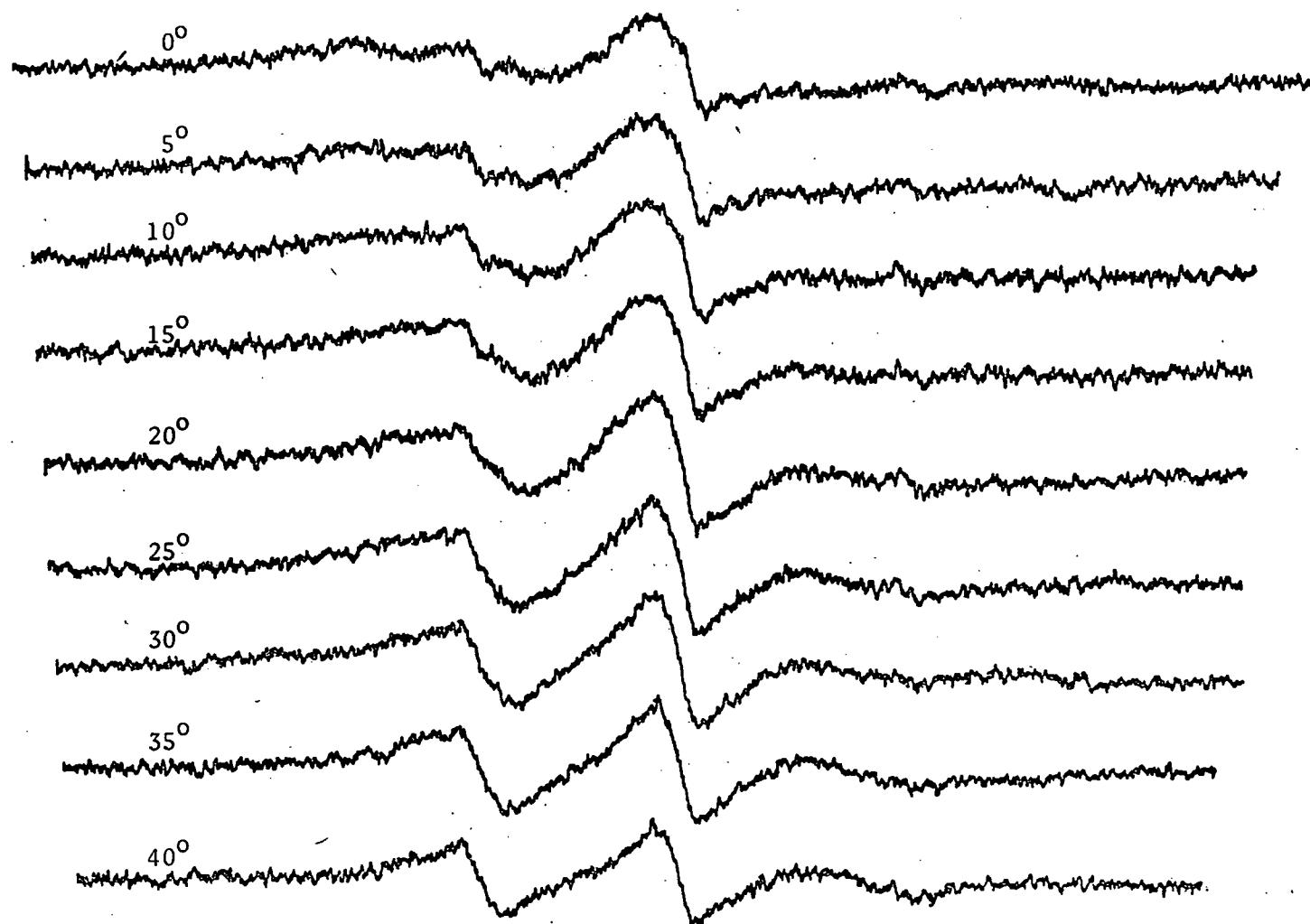
In analogy with the eosin  $\chi$  measurements, yeast were incubated for 20 minutes in 20  $\mu$ M 8-MOP and were then washed prior to UV-A irradiation. Survival curves measured from the resulting colony counts revealed much less killing than was observed in Figure 18, and again no dark recovery was detected. These results indicate that little 8-MOP was strongly bound to the cells. Sensitization must be caused either by extracellular 8-MOP or by 8-MOP which is highly reversibly bound.

#### Spin Label Studies of Membrane Photodynamic Damage

Photodynamic action has been demonstrated both in naturally-occurring biological membranes (Lamola et al., 1973) and in model membrane systems (Anderson and Krinsky, 1973; Anderson et al., 1974). Membrane transport in *E. coli* has been shown to be subject to photodynamic inactivation (Barran et al., 1974) as has been calcium ion uptake and ATPase activity of sarcoplasmic reticulum vesicles (Kondo and Kasai, 1974). Spin label ESR studies have been undertaken in order to examine the effects of membrane photodynamic damage in yeast and to determine how membrane damage is correlated with cell survival.

Membrane spin label spectroscopy relies on the characteristic orientation dependence of the nitroxide ESR spectrum (Keith et al., 1974). The labeled molecules consist of a nitroxide-containing ring structure covalently bound to a membrane-soluble molecule such as hydrocarbon or a fatty acid.

Figure 20. ESR spectra of  $12\text{NS}_{\text{me}}$  in unirradiated diploid yeast before treatment with toluidine blue O.



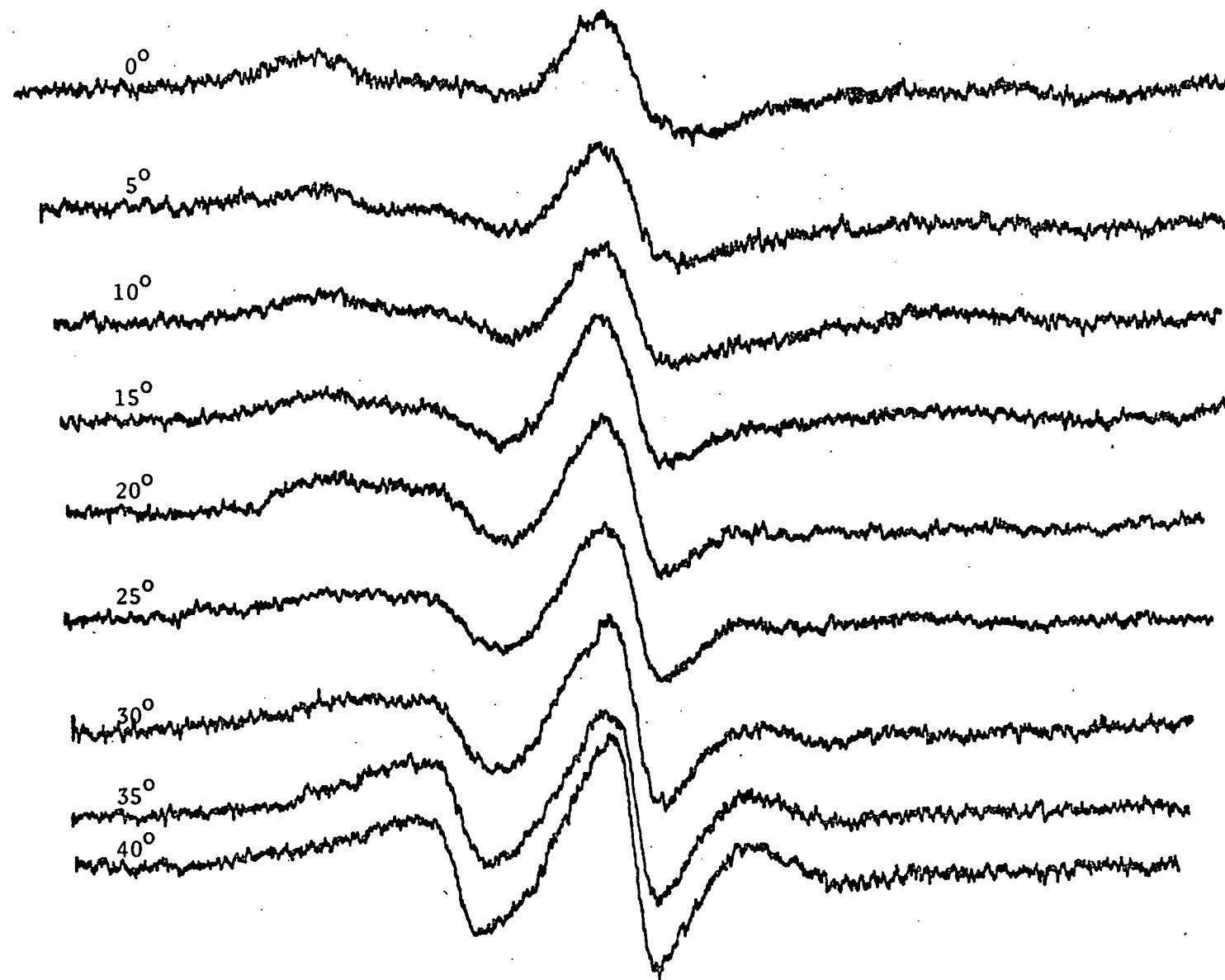


Figure 21. ESR spectra of yeast photodynamically irradiated in the presence of 20  $\mu$ M toluidine blue O for 72 minutes and labeled with  $^{12}\text{NS}_{\text{me}}$ .

Because of the hyperfine interaction of the unpaired electron with the spin-1 nitrogen nucleus in the nitroxide group, the observed ESR signal of labels in dilute solution consists of three absorption lines. The splitting between lines and the position of the midline depend on the orientation of the label molecule with respect to the D.C. magnetic field of the spectrometer (Carrington and McLachlan, 1967), and changing this orientation alters the spectrum dramatically (Libertini and Griffith, 1970; Snipes et al., 1974). If the change in orientation results from sufficiently rapid molecular tumbling of the probe ( $\tau_c < 10^{-9}$  sec), so that more than one distinct orientation is assumed during an excited spin state lifetime, the directional dependence can be partly or totally averaged (Williams et al., 1971). Measurements of the heights and widths of the peaks can then be employed to compute the rotational correlation time of the spin label (see previous progress report). For sufficiently rapid tumbling ( $\tau_c < 10^{-11}$  sec) the averaging is complete and the lines have identical heights and widths (Snipes and Keith, 1970).

The degree of order in biological membranes has been shown to depend on the temperature with order-disorder phase transitions detected at characteristic temperatures (Tourtellotte, 1972). ESR spectra reflect this transition as a discontinuity or a cusp in the temperature dependence (Arrhenius plot) of the tumbling time and related ESR spectral parameters (Raison et al., 1971; Mehlhorn and Keith, 1972). An increase in order results in increased hindrance to spin label motion which impedes motional averaging and thus affects relative line heights and widths (Eletr et al., 1974). The temperature dependence of spin label motion can thus be used to characterize membrane properties and to investigate alterations of these properties.

Y55 yeast were prepared and photodynamically irradiated in the presence of eosin Y as described earlier. The cells were then centrifuged out of the

irradiated suspension and the fatty acid spin label 12 NS<sub>me</sub> was added to the pellet. Labeled yeast were then drawn into a micro-pipet which was sealed for ESR analysis. Adding the label to the cells in this manner favors intercalation of the probe molecules into the plasma membrane, since this is the first nonpolar region which the hydrophobic spin labels encounter when they approach the cells. Photochemical damage to the labels was prevented by labeling the cells after irradiation. ESR spectra were recorded in the range 0°C- 40°C for the irradiated cells and for a control culture which was neither strained nor irradiated. The label 12 NS<sub>me</sub> is expected to localize with the alkyl chain with the attached oxazolidine ring extending into the nonpolar membrane interior. Figures 20 and 21 present the temperature series for control and irradiated cultures, respectively, as labeled with 12 NS<sub>me</sub>. These spectra indicate that spin label motion in the hydrocarbon interior of the membrane is significantly hindered following irradiation under photodynamic conditions. When these experiments were performed with the label 2N19, the spectra indicated that the pooling of spin labels into local impurity regions (see previous progress report) observed in unirradiated cells may be substantially decreased. These results suggest that photodynamic action sensitized by eosin Y increases the rigidity of the membrane interior to some degree while the outer regions experience a "loosening" effect.

Since eosin Y can sensitize membrane alterations even though this sensitizer appears not to be bound to the cells, it may be expected that a sensitizer which does bind might have more striking effects. Optical absorption spectra of yeast stained with 20 µM toluidine blue O in aqueous solution indicate that this dye does interact with yeast. It has previously been shown that toluidine blue sensitizes the photodynamic disruption of the membrane associated electron transport chain in Sarcina lutea (Prebble and Huda,

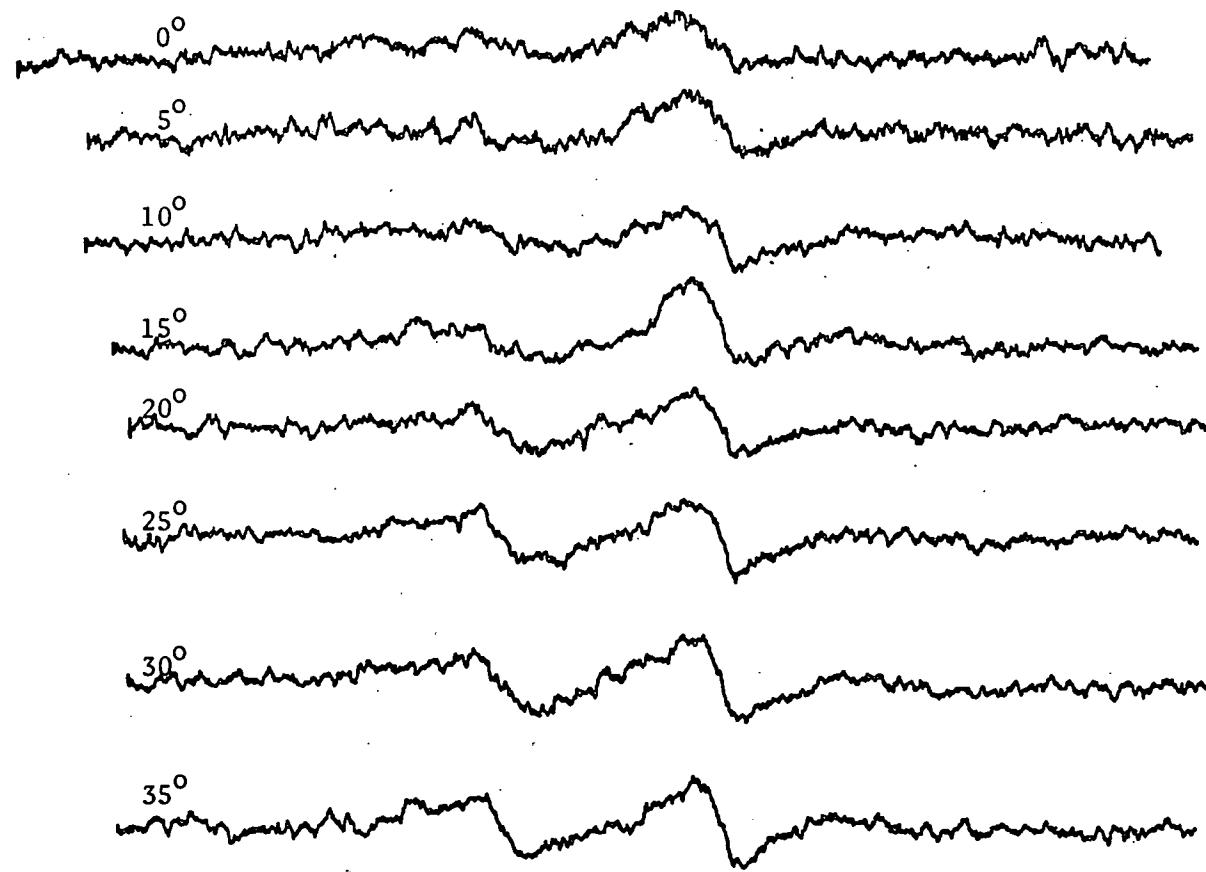


Figure 22. ESR spectra of  $12\text{NS}_{\text{me}}$  in unirradiated diploid yeast before treatment with eosin Y.

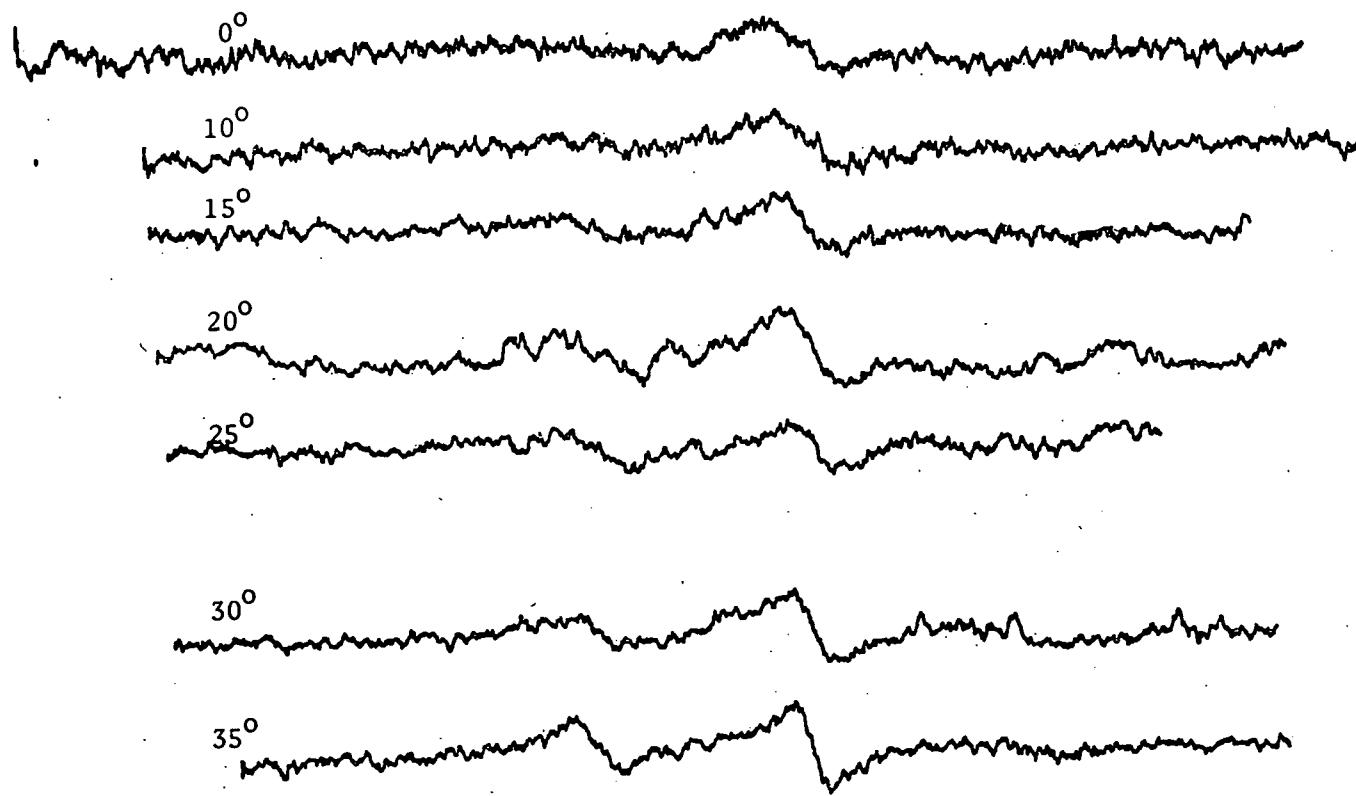


Figure 23. ESR spectra of yeast photodynamically irradiated in the presence of 25  $\mu$ M eosin Y for 2 hours and labeled with  $^{12}\text{NS}_{\text{me}}$ .

1973) as well as the photodynamic disintegration of phospholipid vesicles (Anderson and Krinsky, 1973). Yeast irradiated in the presence of 20  $\mu$ M toluidine blue and oxygen were labeled with  $^{12}\text{NS}_{\text{me}}$  for ESR analysis and aliquots of the same suspension were plated following irradiation to test for survival. The spectra of Figures 22 and 23 indicate that the spin label molecules experience increased hindrance to rotational motion following irradiation, again indicating a significant alteration in membrane properties. The fraction of cells surviving one hour of irradiation in this experiment was at least 100 times smaller than for the same period of eosin-sensitized photodynamic irradiation.

## 6. Related Pulse Radiolysis Studies

Pulse radiolysis and related ionizing radiation studies have been carried out at Michael Reese Medical Center, in collaboration with the research group in the Department of Medical Physics, on problems closely connected with the photochemical work of this program. In particular, we have explored the ability of 8-methoxypsonalen to act as an anoxic radiosensitizer of T7 bacteriophage, the effect of eosin binding to lysozyme on the inactivation of this enzyme by 30 MeV electrons, and the relationship between the initial oxidation products formed by reactions of anion radicals with tryptophan and the corresponding UV photooxidation products. These studies have been carried out jointly by the members of the Biophysics Laboratory at IIT and the following staff members of Michael Reese Medical Center: Dr. J. Ovadia (Director, Department of Medical Physics and Adjunct Professor of Physics, IIT), Dr. A. G. Kepka (Medical Physicist and Adjunct Assistant Professor of Physics, IIT), Dr. J. L. Redpath (Attending Physician), Dr. R. Santus (Research Associate), and Dr. L. Patterson (Research Associate). The IIT graduate students participating in these projects were Mr. D. Becker and Mr. F. T. Tien. A brief summary of the research results follows.

### Radiosensitization of T7 Bacteriophage by 8-Methoxypsonalen

The psoralens are in vivo DNA photosensitizers when irradiated from 320 nm to about 380 nm. Active derivatives such as 8-methoxypsonalen (8-MOP) complex with DNA in the dark and form stable photo-adducts on irradiation; e.g. the review of Musajo and Rodighiero (1972). Musajo et al. (1970) found that 5,8-dihydropsoralen sensitizes the inhibiting effect of  $^{60}\text{Co}$   $\gamma$ -rays on the transmissibility of Ehrlich ascites-tumor cells. Although the role of oxygen was not determined, the DNA photosensitizing properties do not require oxygen, which suggests that psoralens might act similarly for ionizing

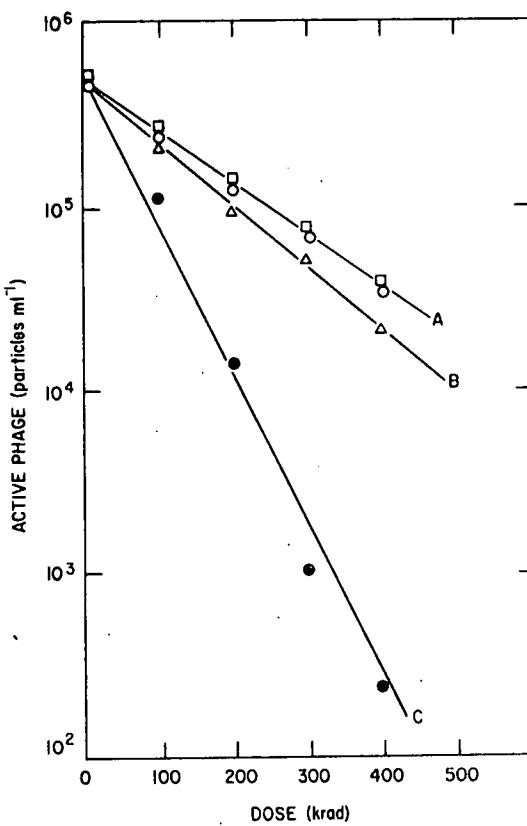


Fig. 24

Irradiation of T<sub>7</sub> phage in nutrient medium; 1.4 krad per pulse, 2 pulses per sec. (A) Oxygen-saturated  $\square$ , oxygen-saturated with 330  $\mu$ M 8-methoxysoralen  $\circ$ . (B) Nitrogen-saturated. (C) Nitrogen-saturated with 330  $\mu$ M 8-methoxysoralen

Condition†	$G_{ln}\ddagger$
$N_2$	0.31
$N_2 + DTT$	0.11
$N_2 + 8\text{-MOP}$	0.77
$N_2 + 8\text{-MOP} + DTT$	0.21
$O_2$	0.26
$O_2 + DTT$	0.24
$O_2 + 8\text{-MOP}$	0.26
$O_2 + 8\text{-MOP} + DTT$	0.36

† 8-MOP indicates 300  $\mu$ M 8-methoxysoralen and DTT indicates 0.1 M dithiothreitol.  
 ‡ Phage inactivated per 100 eV absorbed in DNA.

Inactivation of T<sub>7</sub> phage in nutrient medium by 35 MeV electrons.

Table XII

radiation. The present work was carried out with T7 phage as the test system, where the distinction between direct action and indirect action can be evaluated by introducing radical scavengers in the medium. The irradiation source was a LINAC providing 1  $\mu$ sec pulses of 35 MeV electrons. The phage purification and handling procedures of Redpath and Dewey (1974) were used, and the activity assay was made by plating on *E. coli* B/r. No toxicity to the phage was observed at the 8-MOP concentrations used, and the addition of phage to irradiated medium containing 8-MOP resulted in no inactivation.

The data in Figure 24 show that 8-MOP sensitizes the inactivation of T7 phage irradiated in nitrogen-saturated nutrient medium but has no effect in oxygenated solutions. This result is in marked contrast to the anoxic radiosensitizer of bacterial and mammalian cells, para-nitroacetophenone (PNAP), which mimics oxygen by protecting T7 phage in anaerobic medium and has no sensitizing effect in oxygen (Adams et al., 1971). The G values in the Table XII, in terms of phage particles inactivated per 100 eV absorbed by DNA, are based on the DNA molecular weight or  $25 \times 10^6$  daltons (Blok and Loman, 1973). The protection of T7 phage by oxygen in nutrient medium has been observed by other workers (e.g. the data summary in Dewey 1972). The protection by the sulphydryl reagent, dithiothreitol (DTT), under nitrogen also is consistent with other work (Freifelder, 1965; Van der Schans and Blok, 1970), but the ability of 8-MOP to sensitize in oxygenated medium containing DTT is surprising. In dilute buffer, the presence of 8-MOP protects T7 phage relative to 'blank' runs with oxygen or nitrogen saturation (Figure 25). However, the extent of protection is smaller in nitrogen, indicative of anoxic sensitization of DNA lesions in competition with radical scavenging by 8-MOP.

Blok and Loman (1973) compared yields of single-strand breaks ( $G_{ss}$ ) and double-strand breaks ( $G_{ds}$ ) in T7 phage with inactivation yields ( $G_{in}$ ) and

pointed out that the increase in  $G_{ss}$  from 1.2 in  $N_2$  to 1.6 in  $O_2$  and the increase in  $G_{ds}$  from 0.04 in  $N_2$  to 0.11 in  $O_2$  indicates that oxygen must protect the phage by inhibiting another lesion. They propose that this other lesion is cross-links between different parts of a DNA molecule or between DNA and protein and suggest that the actual yield of cross-links may be considerably higher than previous estimates of about 0.03, because of the difficulty in detecting them. The photochemical studies show that psoralens form DNA cross-links when irradiated in vitro (Dall'Acqua et al., 1970; Dall'Acqua, et al., 1971), suggesting that the presence of 8-MOP promotes this type of lesion. A rough estimate of the yields can be made by applying data previously reported for the ratio of strand breaks to inactivation to our inactivation yields. It is assumed that  $G_{in}$  can be expressed as

$$G_{in} = G_{ss}n_{ss} + G_{ds}n_{ds} + G_p n_p , \quad (3)$$

where the  $n$ 's are the probability that the lesion leads to inactivation and  $p$  refers to cross-links and lesions other than strand breaks. Three simultaneous equations for  $G_{in}$  measured in  $N_2$ ,  $O_2$  and  $N_2$  with 8-MOP present can be solved by assuming: (a) a double-strand break is always lethal ( $n_{ds} = 1$ ); (b) 8-MOP does not alter  $G_{ss}$  or  $G_{ds}$  in  $N_2$ ; (c)  $G_p n_p$  is negligible in oxygenated solutions with or without 8-MOP. The  $G_{in}$  data in the table and the reported values of Van der Schans and Blok (1970):  $G_{ss}/G_{in} = 3(N_2), 5(O_2)$ ;  $G_{ds}/G_{in} = 0.10(N_2), 0.33(O_2)$  lead to  $n_{ss} = 0.13$  and  $G_p n_p(8\text{-MOP})/G_p n_p = 4$ . The model predicts that double-strand breaks are about seven times more lethal than single-strand breaks and that the addition of 8-MOP enhances the formation of lesions categorized as 'cross-links' by a factor of 4. The inhibition of cross-links by oxygen is consistent with earlier work with dry DNA (Hagen and Wellstein, 1965). However, the absence of 8-MOP sensitization in oxygen

supports the assumption that the product of the yield  $G_p$  and lethality  $\eta_p$  can be neglected. In summary, it is proposed that the principal lesions in T7 phage are single-strand breaks produced in high yield with low lethality, double-strand breaks produced in low yield with high lethality, and cross-links accounting for about 50 per cent of inactivation in the absence of oxygen or exogeneous sulphhydryl. The anoxic sensitizing action of 8-MOP is attributed to the promotion of cross-link-type lesions.

#### Radiosensitization of Lysozyme by Dye Binding

The radiosensitization of biomolecules is generally discussed in terms of two processes: conversion of the primary radicals to more damaging or selective species such as radical anions, or secondary reactions of the sensitizer at the primary damage site of the substrate. However, neither provides for the complexing of the sensitizer and its possible effect on the damage pathway. Here we report preliminary results demonstrating that the complexing of eosin to lysozyme enhances the sensitivity of the enzyme to the inactivating attack of  $\text{OH}^\cdot$  and  $(\text{CNS})_2^-$  without significantly increasing the overall reactivity towards these radicals. Lysozyme provides a useful system, because 90 per cent of the inactivation at intermediate pH can be attributed to  $\text{OH}^\cdot$  (Aldrich and Cundall, 1969). The enzyme is protected by t-butanol but not by thiocyanate, because of the selective attack of  $(\text{CNS})_2^-$  on tryptophyl residues, (Adams et al., 1969). Eosin forms a 1:1, non-inhibitory complex with lysozyme (Kepka and Grossweiner, 1973). The concentrations of free eosin, free lysozyme, and the complexed protein in the solutions used were calculated from equilibrium binding constants determined with the spectrophotometric method (Kepka and Grossweiner, 1973; Baugher et al., 1974). The irradiations were performed on Worthington hen egg-white lysozyme and chromatographically-purified eosin in 2 mM, pH 7.0 phosphate buffer, using 25 MeV electrons from

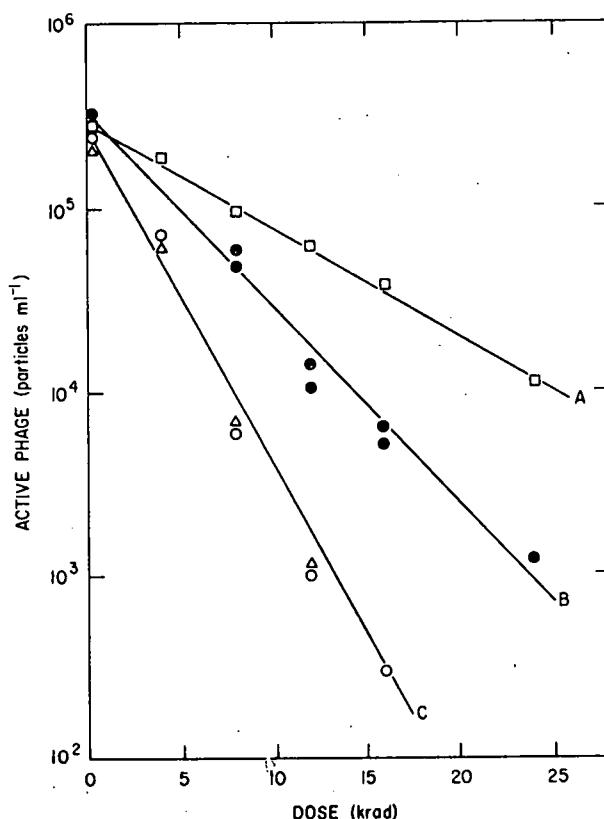


Fig. 25

Irradiation of T<sub>7</sub> phage in 0.06 M phosphate buffer; 2 krad per minute.  
 (A) Oxygen-saturated with 330  $\mu$ M 8-methoxysoralen. (B) Nitrogen-saturated with 330  $\mu$ M 8-methoxysoralen. (C) Nitrogen-saturated O, oxygen-saturated  $\Delta$ .

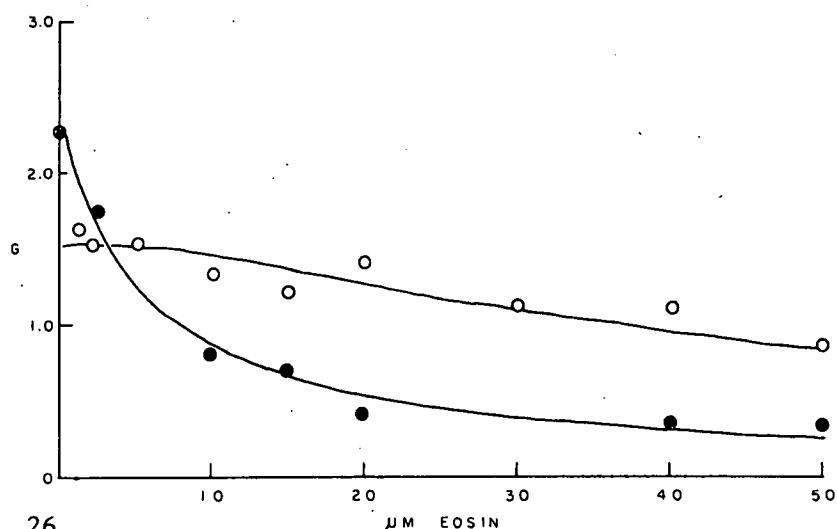


Fig. 26

G values for inactivation of lysozyme (7  $\mu$ M, pH 7.0) in the presence of eosin: O N<sub>2</sub>O-Sat,  
 ● N<sub>2</sub>O-Sat + 10 mM KCNS. The solid lines are based on equations 1a and 1b.

a linear accelerator at 2.5 krads/min. Lysozyme activity was determined with the Worthington Assay Kit based on the Micrococcus lysodeikticus substrate. The analysis of inactivation G values was carried out with a PDP-8/e digital computer, using a least-squares program based on 'steepest descent' to obtain the best fit of the data to equation .

The experimental points in Figure 26 show the effect of adding eosin to 7 $\mu$ M lysozyme, for irradiations in  $N_2O$ -saturated solutions with and without 10 mM KCNS. The overall decrease in  $G_i$  with increasing eosin concentration does not imply that the dye is protective when complexed with lysozyme because of the high preponderance of free eosin. The results were analyzed with the assumed linear relationship:

$$G_i = 5.4 \frac{k_L'[lys] + k_C'[complex]}{k_L'[lys] + k_E'[eosin] + k_C'[complex]} \quad (4)$$

where  $k_L$ ,  $k_E$ , and  $k_C$  are the total rate constants for reaction of  $OH\cdot$  (or  $(CNS)_2^-$ ) with free lysozyme, free eosin, and the complex, and  $k_L'$  and  $k_C'$  are the corresponding rate-constants for inactivation of the enzyme. The unknown constants to be evaluated were reduced from five to three by carrying out comparable irradiations in the presence of 0.1 M NaCl, where eosin binding to lysozyme is negligible. In this case  $k_C' = k_C = 0$ , leading to the best-fit values  $k_L'/k_L = 0.28$  and  $k_E'/k_L = 0.28$  for  $OH\cdot$  attack;  $k_L'/k_L = 0.44$  and  $k_E'/k_L = 1.7$  for  $(CNS)_2^-$ . These results indicate that lysozyme inactivation by  $(CNS)_2^-$  is about 50 per cent more efficient than by  $OH\cdot$  and that free eosin competes with lysozyme for  $(CNS)_2^-$  more effectively than it does for  $OH\cdot$  by an order of magnitude.

The relative rate-constants in equation (4) were scaled by starting the computer fitting at  $k(OH\cdot + LYS) = 4.9 \times 10^{10}$  and  $k((CNS)_2^- + LYS) = 6.6 \times 10^8$

(all  $M^{-1} sec^{-1}$ ), as reported by Adams et al. (1969), and allowing these values to vary as required to minimize the deviations. The best fit obtains with:

$$G_i(N_2O) = 5.4 \frac{1.39[\text{lys}] + 2.36[\text{complex}]}{4.89[\text{lys}] + 1.36[\text{eosin}] + 4.89[\text{complex}]} \quad (5)$$

$$G_i(N_2O, \text{CNS}^-) = 5.4 \frac{2.57[\text{lys}] + 4.08[\text{complex}]}{5.82[\text{lys}] + 10.04[\text{eosin}] + 6.55[\text{complex}]} \quad (6)$$

The plots of 5 and 6 in the figure fit the data with 10 per cent standard deviation.

The analysis shows that the total reactivity of  $\text{OH}^\cdot$  and  $(\text{CNS})_2^-$  with the dye-protein complex is about the same as with free lysozyme. However, the inactivating efficiency of each radical is higher by 60-70 per cent, which is indicative of sensitization induced by the dye. Although the absolute scale of the rate-constants is undetermined in 5 and 6, the comparison with the earlier lysozyme pulse-radiolysis results suggests that they are  $10^{10}$  and  $10^8$ , respectively. On this basis,  $k_E = 1.36 \times 10^{10}$  is in good agreement with  $k(\text{OH}^\cdot + \text{eosin}) = (1.4 \pm 0.4) \times 10^{10}$  reported by Chrysochoos (1967). Furthermore,  $k_L' = 1.39 \times 10^{10}$  in and  $k_L' = 2.57 \times 10^8$  in would be close to  $k(\text{OH}^\cdot + \text{tryptophan}) = (1.2 \pm 0.2) \times 10^{10}$  (Dorfman and Adams, 1973) and  $k((\text{CNS})_2^- + \text{tryptophan}) = 3 \times 10^8$  (Adams et al., 1969), in support of the mechanism proposed by Adams et al. (1969), where both radicals inactivate lysozyme by reacting primarily with Trp 108.

The structure of this complex was investigated by Baugher et al. (1974) with fluorescence methods, in which the proposed eosin-binding site is the 'hydrophobic box' on the enzyme surface adjacent to the active site crevice about 7 Å from Trp 108. The presence of dye may sensitize the enzyme by perturbing the local conformation, thereby increasing the exposure of Trp 108

to the medium, as postulated in connection with a study of the photodynamic inactivation of lysozyme (Kepka and Grossweiner, 1973). It was found that Trp 108 is the only tryptophan residue destroyed and the complexed lysozyme molecules are more sensitive to the inactivating attack of singlet oxygen than the free enzyme. Alternatively, the complexed dye may act as radical trap which reacts first with  $\text{OH}^\cdot$  (or  $(\text{CNS})_2^-$ ) and subsequently oxidizes the nearby Trp 108 site. In either case, our results suggest that the complexing of a sensitizing or protective agent may be a significant factor in its function as a mediator of radiological response.

#### The Pulse Radiolytic Oxidation of Tryptophan by Radical Anions

The initial radiolysis reactions of aqueous tryptophan (Trp) are important in connection with the radiation inactivation of enzymes. In early pulse radiolysis work on lysozyme, Adams et al. (1969) demonstrated that the attack of the OH radical on an essential tryptophyl residue is a major inactivating process. The reaction was identified with the addition of the OH radical to a double bond of the indole ring system, based on the similarity of the transient spectrum with the absorption reported by Armstrong and Swallow (1969) for OH reaction with aqueous Trp. Similar transients have been observed in trypsin (Masuda et al., 1971) and other Trp-containing proteins. However, the reactions of  $(\text{CNS})_2^-$  and other radical anions with aqueous Trp or tryptophyl residues in enzymes give different products (Adams et al., 1972a; Adams et al., 1973), which have not been identified. The transient absorptions formed by the reactions of  $(\text{CNS})_2^-$ ,  $\text{Br}_2^-$ ,  $\text{I}_2^-$  and  $\text{CO}_3^-$  with Trp in neutral or alkaline solutions have band maxima near 330 nm and 510 nm, which closely resemble the spectra obtained by UV flash photolysis of Trp (Grossweiner et al., 1963; Joschek and Grossweiner, 1966), where the hydrated electron is a co-product. The radical was subsequently identified with the neutral species

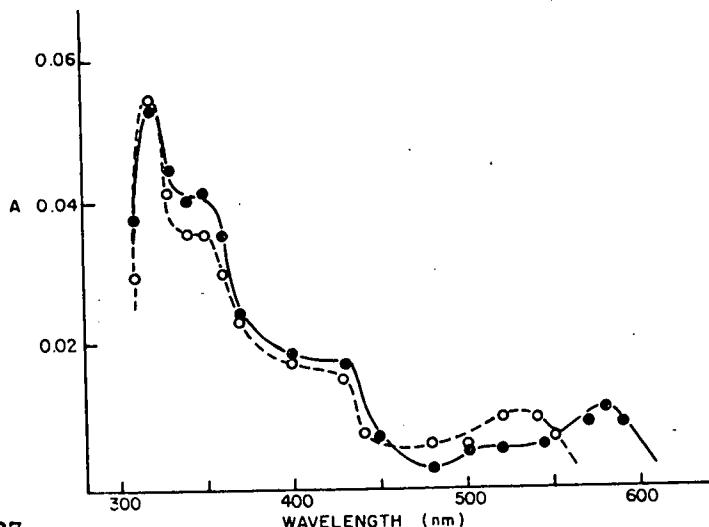


Fig. 27

Pulse radiolysis transient spectra with nitrous oxide saturation.  $\circ$  800  $\mu\text{M}$  tryptophan, 20  $\mu\text{sec}$  delay;  $\bullet$  800  $\mu\text{M}$  1-methyltryptophan, 20  $\mu\text{sec}$  delay. Dose/pulse = 1 krad, pH 7.

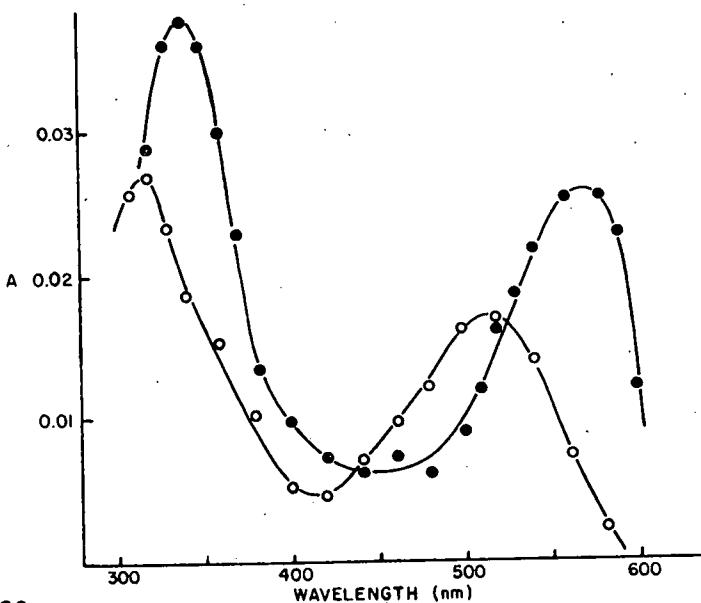


Fig. 28

Pulse radiolysis transient spectra with nitrous oxide saturation in the presence of 0.1 M  $\text{Br}^-$ .  $\circ$  800  $\mu\text{M}$  tryptophan, 50  $\mu\text{sec}$  delay;  $\bullet$  800  $\mu\text{M}$  1-methyltryptophan, 20  $\mu\text{sec}$  delay. Dose/pulse = 1 krad, pH 7.

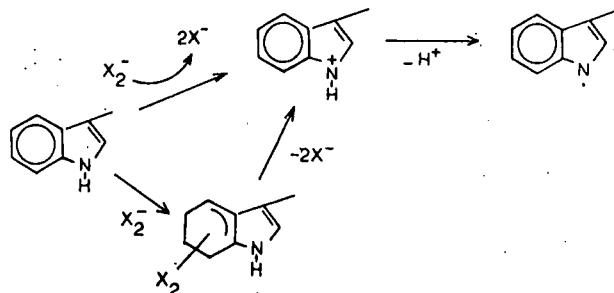
(Trp) by Santus and Grossweiner (1972), who showed that both Trp in strongly acidic solutions and 1-methyltryptophan (1-MeTrp) from pH 0-12 give a different transient with band maxima near 340 nm and 580 nm, attributed to the corresponding radical cations  $\text{Trp}^+$  and  $1\text{-MeTrp}^+$ . The cation spectra are similar to the product of  $\text{Cl}_2^-$  attack on Trp at pH 1.7, where the longer wavelength band is shifted to about 580 nm.

This short study was undertaken to explore the possibility that the anion radical reaction products with Trp are the same as obtained by photochemical oxidation. Parallel pulse radiolysis measurements were made on Trp and 1-MeTrp to differentiate between the neutral and cationic species. The apparatus located at the Radiation Research Laboratories of Carnegie-Mellon University was used for this work to take advantage of the good sensitivity of the computerized system, (Patterson and Lilie, 1974). The irradiations were carried out with 0.5  $\mu$ sec pulses of 2.8 MeV electrons at 0.5-1.5 krads per pulse. The pulse dose was determined by irradiating  $\text{N}_2\text{O}$ -sat 0.01 M KCNS taking  $G[(\text{CNS})_2^-] = 6.0$  and  $\epsilon[(\text{CNS})_2^-] = 7600 \text{ M}^{-1} \text{ cm}^{-1}$  at 480 nm (Baxendale et al., 1968). The initial  $(\text{CNS})_2^-$  yield was used to determine the molar absorbance of the Trp radicals by assuming complete scavenging and extrapolating the radical transient absorptions to the initial time of pulse application.

The transient spectra from pulse radiolysis of 800  $\mu\text{M}$  Trp and 1-MeTrp in  $\text{N}_2\text{O}$ -sat, pH 7 phosphate buffer (1 mM) are shown in Figure 27. The Trp transient attributed to the OH adduct with overlapping bands of decreasing intensity at 320 nm, 360 nm and 420 nm is in excellent agreement with the work of Armstrong and Swallow (1969). The similar spectrum from 1-MeTrp supports the identification, because this absorption should not be sensitive to methyl substitution on the indole N atom. The weak band near 580 nm from 1-MeTrp suggests a small contribution from the oxidation product  $1\text{-MeTrp}^+$ . The addition

of 0.1 M  $\text{Br}^-$  led to the spectra in Figure 28; equivalent results were obtained by adding 0.1 M  $\text{CNS}^-$ . The 510 nm band from Trp is attributed to the  $\text{Trp}^\cdot$  radical and the 570 nm band from 1-MeTrp to  $1\text{-MeTrp}^+$  by comparison with the flash photolysis results (Santus and Grossweiner; 1972). (This reference cites prior work on UV-irradiation of Trp in neutral and acidic rigid media in which corresponding bands near 500 nm and 600 nm were observed.) Moan and Kaalhus (1974) reported the formation of the 600 nm  $\text{Trp}^+$  absorption by irradiating Trp with UV or X-rays in acidic, frozen aqueous solutions, in support of the present pulse radiolysis results.

The present results indicate that one-electron oxidation is the major mode of radical anion attack on tryptophyl residues in enzymes. The actual mechanism may involve direct electron transfer or take place via adduct formation:



It is interesting to note that the reactions of  $(\text{CNS})_2^-$ ,  $\text{Br}_2^-$ ,  $\text{CO}_3^-$  and  $\text{Cl}_2^-$  on tyrosine lead to a similar transient band at 410 nm (Adams et al., 1972a), which was also observed in the pulse radiolysis of RNase (Adams et al., 1972b). This absorption agrees with the phenoxy radical spectrum obtained by UV flash photolysis of aqueous tyrosine (Grossweiner et al., 1963) and RNase and insulin, which contain tyrosine but not tryptophan (Volkert and Grossweiner, 1973), strongly suggesting a similar electron transfer mechanism for the radical anion attack on tyrosyl residues.

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7, 207.

Project Activity

The expenditure of scientific effort and funds was consistent with the technical program in the Renewal Proposal and the current budget.

Scientific Salaries

Dr. Leonard I. Grossweiner (Professor): 1 month Summer 1975; 1 month Summer 1976; 10% of time 1975-76 academic year.

Dr. Gerald E. Cohn (Assistant Professor): 1 month Summer 1975; 25% of time 1975-76 academic year.

Dr. Joon Lee (Research Associate): 100% of time 9/1/75-6/30/76

Foreign Travel

none

Non-Salaried Student Participants

Mr. David S. Becker: IIT Tuition Scholarship support, 1976-76 academic year.

Mr. Albert Tien: Graduate Teaching Assistant, 1975-76 academic year.

Miss Hsiu-Yu Tseng: Graduate Teaching Assistant, sem.-1, 1975-76 academic year.

Reporting of Research

A. Journal Articles

1. "Photosensitizing Properties of N-Formylkynurenine", P. Walrant, R. Santus, and L. I. Grossweiner; Photochem. Photobiol. 22, 63 (1975). C00-2217-8
2. "Photochemical Inactivation of Enzymes", L. I. Grossweiner; Current Topics in Radiation Research Quarterly, 11, 000 (1975). C00-2217-7
3. "Ultraviolet and Photodynamic Inactivation of Lysozyme", L. I. Grossweiner; Acad. Brasileira de Ciencias 45, 45 (1975).
4. "Flash Photolysis of Enzymes", L. I. Grossweiner, A. G. Kaluskar, and J. F. Baugher; Int. J. Radiat. Biol., 28 000 (1976). C00-2217-9
5. "Lethality of Primary Lesions in E. Coliphage", L. I. Grossweiner; Radiat. Res., submitted C00-2217-10
6. "Laser Flash Photolysis of Aqueous Tryptophan", F. Dudley Bryant, R. Santus, and L. I. Grossweiner; J. Phys. Chem., 79, 2711 (1975). C00-2217-11
7. "Photodynamic Sensitization by 8-Methoxysoralen via the Singlet Oxygen Mechanism", W. Poppe and L. I. Grossweiner; Photochem. Photobiol. 22, 217 (1975). C00-2217-12
8. "Ultraviolet Inactivation of Papain", J. F. Baugher and L. I. Grossweiner; Photochem. Photobiol. 22, 163 (1975). C00-2217-13

B. Meetings

American Society for Photobiology, Louisville, Ky., June 22-26, 1975.

1. "Laser Flash Photolysis of Aqueous Tryptophan", F. Dudley Bryant, R. Santus and L. I. Grossweiner.

E. J. Hart Conference on Aqueous Radiation Chemistry and Solvated Electrons, Argonne National Laboratory, July 7-9, 1975

2. "The Role of the Hydrated Electron in the Photochemical Inactivation of Enzymes", L. I. Grossweiner. (Invited)

VIII Int. Conf. on Photochemistry, Edmonton, Canada, August 7-12, 1975.

3. "Photochemical Inactivation of Enzymes", L. I. Grossweiner. (Invited)

American Society for Photobiology, Denver CO, Feb. 16-20, 1976

4. "Photochemistry of Papain", J. F. Baugher and L. I. Grossweiner.

5. "Photochemical Inactivation of Enzymes", L. I. Grossweiner. (Invited)

American Physical Society Meeting, March 29 - April 1, 1976

6. "Photo-irradiation Damage in Yeast Sensitized by 8-Methoxysoralen", G. E. Cohn and H. Y. Tseng, S. C. Weiss and L. I. Grossweiner.

7. "Lethality of Primary Lesions in E. Coliphage", L. I. Grossweiner.

Radiation Research Society, June 27 - July 2, 1976

8. "Laser Flash Photolysis of Enzymes", J. F. Baugher and L. I. Grossweiner.