PHOTOCHEMICAL YIELDS IN RIBONUCLEASE AND OXIDIZED GLUTATHIONE IRRADIATED AT DIFFERENT WAVELENGTHS IN THE ULTRAVIOLET

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ABSTRACT The quantum yields for the disruption of various amino acids in glutathione and ribonuclease by 229, 254, 265, and 280 nm UV photons have been determined. The results of the measurements on the destruction of tyrosine and histidine and the loss of enzymic function in RNAse and the disruption of cystine in both compounds lead to the following conclusions: (*a*) The photodestruction of some and perhaps many constituent amino acid residues does not cause RNAse inactivation. (*b*) Contrary to the basic premise of proposals made by other authors, the photochemical yields of constituent residues in a protein are not the same as that for the same amino acids in solution alone—the difference is a function of the exciting wavelength. Further, the extent of histidine destruction varies by a large factor among three proteins. (*c*) Consistent with previous predictions, the present results show that photons absorbed in the aromatic residues of RNAse cause the disruption of cystines elsewhere in the enzyme. (*d*) Although cystine disruption appears to be the most prevalent mode of RNAse inactivation by photons of the four wavelengths studied, some of the minor mechanisms leading to loss of enzymic function may vary with the UV energy.

INTRODUCTION

It is of obvious photobiological interest to determine whether UV energy absorbed in a protein can destroy only the residue initially excited or whether biological function can be impaired as a result of damage produced some distance away following energy transfer. Such knowledge is also of more general importance since quantitative determinations of energy transfer can provide useful insight into the organization of proteins and of the consequent interactions between constituent residues. Accordingly, the present experiments were designed to evaluate the validity of conflicting theories concerning the extent and importance of energy transfer in the UV inactivation of enzymes.

A number of years ago two independent proposals were made by Setlow and Doyle (1) and Augenstein and co-workers (2–4) concerning the importance of cystine disruption in the irradiation inactivation of proteins. These proposals differ in the mechanisms they associate with enzyme inactivation and more basically in the extent to which they propose that specific, as opposed to random, breakage of cystines is involved. Subsequently, Luse and McLaren proposed that energy transfer is of no consequence in enzyme inactivation and assumed that a given amino acid has the same photochemical behavior whether it is a residue inside a protein, in a small peptide, or free in solution (5, 6).

A number of publications have shown that cystine disruption is not only correlated with the UV inactivation of a number of enzymes (3, 4, 7), but also that it must be a critical step in the loss of enzymic function (8). Of particular pertinence to this study, it was shown in earlier reports that cystine disruption in RNAse by 254 nm light does not occur at random: using a ^{14}C-labelled sulfhydryl reagent it was found that one, or at most two, of the four constituent cystines was disrupted to any significant extent by doses sufficient to destroy 50% of the activity (4). Further, the photodestruction of residues by 254 nm light in the tripeptide glutathione and three out of four proteins (7) is not consistent with the simplified scheme of McLaren and Luse.

To explain this demonstrated specificity of cystine disruption, Augenstein et all predicted that energy transfer to aromatic residues either immediately adjacent to, and/or bonded to a cystine could cause the latter's disruption (8), and Augenstein and Chaudhuri anticipated that significant electronic interactions occur between some tyrosines and cystines in RNAse on the basis of their spectroscopic studies (9). Recently Cowgill has interpreted the fluorescence quenching in simple compounds containing disulfide and sulfhydryl groups as evidence for interaction between aromatic residues, and these groupings (10). Dose has also claimed that cystines can be disrupted by energy absorbed in aromatic groups in amino acid mixtures[1]; however, he, like Luse and McLaren, claims that these interactions occur with the same yield independent of the environment (11).

We designed the present experiments to investigate the following assumptions implicit in the various proposals. (*a*) Does the photodestruction of just any amino acid residue in a protein cause enzyme inactivation? Setlow and Doyle did not specifically take a stand on this question but merely said enzyme inactivation is correlated with cystine disruption (1). Augenstein specified that enzyme inactivation should be caused by the disruption of only specific cystines (2); whereas McLaren and Luse first said the destruction of any residue destroyed enzyme activity (5), but later agreed that probably the destruction of some residues is not critical (12). (*b*) How much does the photochemical response of a given amino acid vary? The analyses of McLaren and Luse and also of Dose (footnote 1 and reference 11)

[1] K. Dose. Submitted for publication.

were based on the stated premise that the yields for the destruction of a given amino acid are the same "inside" and "outside" of a protein. By contrast, Augenstein initially proposed that some cystines are more susceptible to disruption than others (2). (*c*) Can photons absorbed by the aromatic residues cause the destruction of the integrity of other residues? As noted above, Augenstein et al. predicted the disruption of specific cystines as a result of energy transfer. Dose also later proposed that aromatic-cystine interactions occur, but with a constant yield of photodestruction for the residues involved; and McLaren claims energy transfer is of no consequence in enzyme inactivation. (*d*) Is the mechanism of enzyme inactivation the same at various wavelengths? Setlow and Doyle based their predictions of the importance of cystine disruption in enzyme inactivation on studies conducted at various wavelengths.

To investigate these various possibilities further, we measured the yields of various photoproducts from RNAse and glutathione irradiated by four wavelengths of UV. Specifically, we have undertaken the following experiments: (*a*) redetermined the quantum yields for enzyme inactivation of RNAse by light having a wavelength of 229, 254, 265, or 280 nm; (*b*) performed amino acid analyses on the irradiated samples of both RNAse and the model compound oxidized glutathione;[2] (*c*) measured in both compounds the production of SH groups which can be titered with *p*-chloromercuribenzoate (*p*CMB), the production of cysteine as a result of cystine breakage, the total loss in identity of cystines, and the cysteic acid formed at the various wavelengths; and (*d*) analyzed specially for histidine in RNAse by carrying out enzymic hydrolysis following irradiation. The four wavelengths were chosen on the following bases (see Table I): at 280 nm over 90% of the light is absorbed in the tyrosine residues of RNAse and further, the lowest-lying absorption peak of RNAse is at 277 nm; Setlow and Doyle reported that the enzyme inactivation yield at 265 nm is only one-third that at 254 nm (1); the fraction of the light absorbed by both cystine and phenylalanine is maximal at 254 nm; and at 229 nm the peptide bonds of RNAse absorb almost 50% of the light and the aromatics and cystines are excited to the second excited energy levels.

By choosing RNAse we have investigated mechanisms in a cystine-containing enzyme. Clearly other residues will be much more crucial in the inactivation of a protein which contains no cystine.

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[2] Oxidized glutathione is two γ-glutamyl-cysteinyl-glycine tripeptides held together by a disulfide bond: i.e.,

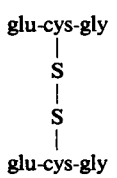

TABLE I

SPECTRAL DATA ON RNAse

Wavelength	Fraction of the light absorbed by constituent		
	Tyrosine	Phenylalanine	Cystine
nm			
229	0.41	—	0.02
254	0.51	0.12	0.33
265	0.80	0.05	0.14
280	0.93	—	0.06

At 254 and 265 nm the molar extinction coefficient for RNAse agrees with the sum of the absorbances for the constituent amino acids. At 280 nm the observed RNAse value of 8500 is much larger than the sum (7700) for the amino acids: in the above calculation of the fraction of light absorbed by the various moieties we have used the value of 7700. At 229 nm, we could not calculate an extinction coefficient because of the large variations in the absorbance of peptide bonds connecting different amino acid residues. Hence at 229 nm we have used the observed value.

EXPERIMENTAL METHODS AND MATERIALS

Preparation of Solutions

Ribonuclease (RNAse) (mol. wt. 13,680) solutions of approximately 35 or 70 μM were made by dissolving either 15 or 30 mg of lyophilized phosphate-free material from Worthington Biochemical Corp., Freehold, N.J. in 30 ml of 0.05 M phosphate buffer at pH 5.0. Since we have observed a variable moisture content in the lyophilized material, we determined the exact concentration by measuring the absorbance at the wavelength used for irradiation: the molar extinction coefficients at the four wavelengths were measured separately using material which had been dried for at least two days over P_2O_5. Solutions containing 1.62 and 0.81 mM oxidized glutathione were prepared by dissolving 30 and 15 mg of oxidized glutathione obtained from Boehringer, Mannheim, Waldhof (West Germany) in 30 ml of 0.05 M phosphate buffer at pH 5.0.

Irradiations

The UV source was a 250-W GE medium-pressure lamp (General Electric, Schenectady, New York): the desired wavelength of light was obtained by using a suitable interference filter having a 7 nm "band width". Irradiations were done at 0–4°C with constant striring in an apparatus described previously (8). The exposures at 280, 265, and 254 nm were carried out on 70 μM RNAse or 1.62 mM oxidized glutathione, but because of the large extinction coefficients at 229 nm, solutions only one-half as concentrated were irradiated at the latter wavelength.

To avoid the possible complications of high dose rates described previously (3), we utilized fluxes sufficiently low so that the t_{37} (time required to inactivate 63% of the activity) values were all more than five hours. The flux incident on the front face of the 1.5 cm thick cuvette was determined by a standard procedure using a uranyl oxalate actinometer (12): at the four

wavelengths the values were 5.4×10^{-7} (280), 8.9×10^{-7} (265), 8.0×10^{-7} (254), and 2.4×10^{-7} (229) Einsteins-hr^{-1}-cm^{-2}.

The methods used for determining accumulated doses in our biochemicals and calculating quantum yields for the loss of enzymic activity and the loss and production of individual amino acids were described previously (7): briefly, we irradiated for sufficiently long periods of time so as to destroy up to 70% of the enzymic activity. The log fraction activity remaining was plotted as a function of dose: in all cases, a single exponential was observed. To determine the yield values we combined the data from at least three experiments: the rms deviation of the aggregate points was 5–10%. A comparable procedure was used for determining the yields of amino acid destruction in duplicate or triplicate runs. The values of the increase in SH groups which can be titered per molecule inactivated were obtained from plots of the increase in titrable SH groups vs. loss in enzymic activity (see 4, 8) for at least duplicate experiments. The rms deviation of the aggregate points was 5% or less.

Determination of Biochemical Changes

The methods utilized for determining the residual RNAse activity (13) and the residual amino acid contents (14) have already been described (7). The main change from earlier procedures was in adapting the following special method (15) for histidine analysis.

The presence of phosphate hinders the enzymic hydrolysis of RNAse by Pronase during preparation for histidine analysis. Thus, for this portion of the experiment irradiation of RNAse was carried out in 0.05 M acetate buffer of pH 5.0. Known volumes (4 ml for 229 nm irradiation and 5 ml for the other three wavelengths) of the irradiated and unirradiated solutions were carboxymethylated (trimethylamine was used to raise the pH) and lyophilized. The samples irradiated at 229 nm were dissolved in 1.1 ml of 5×10^{-4} M $CaCl_2$ solution and mixed with 0.3 mg of Pronase (Calbiochem, B grade) in 0.1 ml of the $CaCl_2$ solution. The samples irradiated at the other three wavelengths were dissolved in 1.8 ml of 5×10^{-4} M $CaCl_2$ and 0.5 mg of Pronase in 0.2 ml of the $CaCl_2$ solution was added. The pH was raised to 8.0 with NaOH before sealing *in vacuo*. After 2 hr digestion at 38°C the pH decreased appreciably so we reopened the vial and readjusted the pH to 8 before sealing again under vacuum. After 5 days digestion at 38°C, the samples were lyophilized and analyzed for histidine: in the unirradiated samples we could titrate about 75% of the theoretically expected amounts.

RESULTS

Oxidized Glutathione

The results for irradiation of glutathione are given in Table II. The values of $\phi_{1/2\ cys}$ and ϕ_{SH} are a little larger than those recently reported from this laboratory (7) for glutathione irradiated with 254 nm light at room temperature under nitrogen and the value of ϕ_{CMC} is less. Since our solutions contained normal aeration, these changes of about 20% are to be expected. The ϕ_{NH_3} values for all except 229 nm light is relatively large, but we have noted previously that the values for radiation-produced ammonia are much greater for samples which have undergone acid hydrolysis than for those which have just been irradiated but not hydrolyzed. We could detect no cysteic acid formed at any of the four wavelengths used. This is consistent with the earlier observations that none was produced by 254 nm light in solutions which had been bubbled extensively by nitrogen.

The values in column 2 of Table II clearly indicate that $\phi_{1/2\ cys}$ is not independent of the wavelength used for the irradiation. There are two reasons for expecting that the $\phi_{1/2\ cys}$ at 229 nm should be different from the values at the other three wavelengths: (*a*) 229 nm photons should produce excitation to excited states other than the lowest excited levels of the cystine (note that glutathione has no aromatic amino acids), and (*b*) about 70% of the 229 nm light is absorbed by the peptide bonds, whereas at the other three wavelengths practically all of the absorption should be by the constituent cystine residues themselves. The values of $\phi_{1/2\ cyst}$ at

TABLE II

PHOTOCHEMICAL DATA FOR OXIDIZED GLUTATHIONE

Wavelength of the light used	$\phi_{\frac{1}{2}cys}$	ϕ_{SH}	ϕ_{CMC}	ϕ_{NH_3}
nm				
229	0.06*	0.03	0.02	0.03
254	0.39	0.14	0.08	0.13
265	0.44	0.12	0.11	0.15
280	0.25	0.07	0.06	0.09

Column 2, The quantum yields for the actual disruption of half cystines: all cystines are converted to the S-carboxymethyl form prior to the normal amino acid analysis procedure. Column 3, The quantum yields for the formation of —SH groups as determined by *p*CMB. Column 4, The quantum yields for cysteine formation as determined by amino acid analysis after the —SH groups have been carboxymethylated with iodoacetic acid. Column 5, The quantum yield for the production of NH_3 as measured after hydrolysis in the regular amino acid analysis.

* This value is calculated to be 0.22 for those 229 nm photons absorbed *only* in the cystines. This calculation is based upon the molar extinction coefficient for cystine in solution by itself inasmuch as the absorption of individual peptide bonds varies appreciably and further the peptide bond between two specific amino acids may have extinction coefficients differing by as much as 30% depending upon whether it is present in a dipeptide or tripeptide (16, 17).

254, 265, and 280 nm must reflect cystine disruption by photons adsorbed directly by this moiety since peptide bond absorption in this region is very small; whereas, at 229 nm the $\phi_{1/2\ cys}$ calculated for photons absorbed directly in the cystines is 0.22 (see the footnote in Table II).

At present we cannot account for all of the radiation products. Since ϕ_{SH} (titration with *p*CMB) and ϕ_{CMC} (titration with iodoacetic acid) are almost identical for irradiation at 265 and 280 nm (and perhaps 229 nm) we conclude that we have not produced much C—S—S—H by cystine disruption at these wavelengths. However, both ϕ_{SH} and ϕ_{CMC} are much smaller than the yield for the loss of one-half cystine by all reactions ($\phi_{1/2\ cys}$) and even in this tripeptide we cannot account for the difference on the basis of the formation of ammonia, cysteic acid, and

other compounds containing SH groups. It should be noted that with irradiation at 254, 265, and 280 nm at least two other ninhydrin-positive fragments are found which do not appear at the position of normal amino acids in the analyzer. These fragments are not observed following 229 nm irradiation, but small amounts of serine are detected ($\phi_{ser} < 0.005$). Further, we could not find any evidence of products such as cysteic acid and/or cysteine sulfinic acid which might be formed by the oxidation of cystine; however, we could not rule out the possibility that the latter compound is formed but lost during the acid hydrolysis owing to the presence of the other amino acid residues (18). Neither alanine nor any other amino acid is formed to any significant level from irradiation at all four wavelengths.

Ribonuclease

Ferrini (15) and Ferrini and Zito (19) reported that they could detect a loss of histidine in UV-irradiated lysozyme after enzymic hydrolysis with pronase but not when 6 N HCl was utilized for hydrolyzing the peptide bonds. Apparently, under highly acid conditions histidine is reformed from the irradiation products. Further, these workers found that in lysozyme, which contains only one histidine per molecule, the loss of histidine occurs at the same rate as the loss of enzymic activity.

The situation is different with RNAse since we find no loss of histidine following irradiation at 254, 265, or 280 nm. At 229 nm there is a reduction in residual histidine but the fractional loss does not parallel the fractional loss of enzymic activity. This latter situation is similar to that in carboxypeptidase A where 254 nm UV light does destroy constituent histidines, but at a rate different from that for the change of either peptidase or esterase activity (20).

The yields for the loss and production of different moieties are given in Table III. When the irradiated and unirradiated protein solutions were analyzed directly for NH_3 in the amino acid analyzer *without hydrolysis*, no appreciable ammonia could be detected except at 229 nm. However, when RNAse was hydrolyzed with 6 N HCl, 17 NH_3 molecules were produced per molecule of RNAse whether irradiated or unirradiated. On this basis, it appears that little or no NH_3 is produced by any of the three longest wavelengths investigated where peptide absorption is negligible. The NH_3 which is measured during the normal amino acid analysis must be produced from side chain deamination during the 6 N HCl hydrolysis procedure since there are 10 asparagines and seven glutamines in RNAse.

The yields for enzyme destruction (ϕ_E) are listed in Table III along with the sum of the yields ($\Sigma\phi_i$) for the destruction of all of the individual amino acids. At all four wavelengths, an average of one to two disulfide bonds are disrupted for each enzyme molecule inactivated (see ϕ_E and $\phi_{1/2\ cys}$ in Table III). Table III also contains the quantum yields for destruction of half-cystine (column 5) calculated on the basis of those photons absorbed by cystines alone (see Table I). There are

considerable differences at 229 and 280 nm between the values in RNAse and those obtained in the model compound oxidized glutathione. The difference between the yields for the two molecules is the smallest for irradiation at 254 or 265 nm where the fraction of the light absorbed by cystine is the greatest.

Our value of $\phi_E = 0.030$ at 254 nm agrees well with a previous report from this laboratory (3) and is slightly higher than the values of 0.024 and 0.027 given by Setlow and Doyle (1) and by Luse and McLaren (6), respectively. At 280 nm we agree almost exactly with Setlow and Doyle's value, but at 265 nm our yield is more than

TABLE III

PHOTOCHEMICAL DATA FOR RIBONUCLEASE

Wavelength of the light used	ϕ_E	$\Sigma \phi_{i,RNAse}$	ϕ_{1cys}	$\phi_{1cysRNAse}/f_{cys}$	ϕ_{SH}	ϕ_{CMC}	ϕ_{Tyr}	ϕ_{NH_3}
nm								
229	0.010	0.026	0.026	1.3	0.010	—	0.013	0.006
254	0.030	0.061	0.121	0.37	0.046*	0.038	—	—
265	0.019	0.022	0.043	0.31	0.026*	0.019	—	—
280	0.007	0.016	0.024	0.40	0.008*	0.006	<0.004	—

Column 2, The quantum yields for the loss of enzymic activity. Column 3, The sum of the quantum yields of all amino acid residues destroyed: cystine is treated as one residue. Columns 4, 6, 7, The quantum yields similar to those found in columns 2, 3, and 4 in Table II. Column 5, These values were obtained by dividing the values in column 4 by the values in column 4 of Table I. Column 8, The quantum yields for the loss of tyrosine as determined by normal amino acid analysis. Column 9, The quantum yield for the production of NH_3 as measured by amino acid analysis of the protein without hydrolysis.

* The three values shown were obtained using material purchased three years ago, the remaining values are for material from lot No. RAF6JA (Some of the quantum yields vary a little between samples (21).

twice what they measured. Possible reasons for this latter discrepancy are discussed in a companion report (21).

When trypsin was irradiated with 254 nm light the amount of enzymic inactivation depended upon the treatment after irradiation. This was ascribed to the formation of "damaged" molecules which could be rendered "inactive" by exposure to urea or *p*CMB but put back into an active conformation by initial exposure to substrate (8). This same behavior was not observed, however, in RNAse (3, 4). In the present experiments, we observe that if RNAse is left in acetate buffer at room temperature for a few hours following irradiation with 254 nm light, the amount of inactivation produced by a given dose is reduced. If it is left in acetate buffer containing *p*CMB, however, following irradiation such reactivation is not observed. But at the other three wavelengths no reactivation is found in either acetate buffer

or the *p*CMB solution. This difference from the previous reports (4) is attributed in a companion publication to differences in the sample preparations used (21).

DISCUSSION

The present data provide valuable insight into the questions which prompted this investigation. Thus, this section is organized around the answers to those questions.

(*a*) *Photodestruction of just any Amino Acid Residue in a Protein does not Necessarily Cause Enzymic Inactivation*

Initially McLaren and Luse proposed that the quantum yield for enzyme inactivation should be the sum of the yields for destruction of all the constituent residues (5). In contrast to this, we find that at all four wavelengths the sum of the observed quantum yields for the loss of various individual residues is greater than the quantum yield for RNAse inactivation (at 229, 254, and 280 nm the values are two to three times larger). Hence, some constituent residues, perhaps many, can be destroyed without the loss of RNAse activity.

At 254 and 265 nm no amino acids other than cystine are destroyed to a measureable extent. Further, although tyrosine is lost at 280 nm in addition to cystine, the yield for the former is much less than ϕ_E. Thus, at least at these three wavelengths cystine disruption must be the most important, perhaps almost the only cause, of RNAse inactivation. This point, of course, has been emphasized in previous publications (3, 4, 8) where it was reported that the yield for trypsin and RNAse inactivation closely parallels that for either ½ cystine destruction or increased —SH titer. However, $\phi_{1/2\,cyst} > \phi_E$. Thus, as already mentioned, presumably inactivation is associated with the disruption of specific cystines rather than random destruction (4).

(*b*) *Yields for the Destruction of a Given Amino Acid are not the same "Inside" and "Outside" a Protein, May Differ by more than a Factor of 1000 Between Proteins, and Vary Appreciably with Wavelength*

The quantum yield for the loss of half cystines in RNAse, calculated on the basis of those photons absorbed by cystines alone (column 5, Table III) differ considerably from those in oxidized glutathione (column 2, Table II). These values for RNAse are calculated on the assumption that cystine absorption inside RNAse is the same as for cystine in solution at pH 5. This assumption is reasonable since the molar extinction coefficient, $\epsilon = 3700$, at 254 nm for the lyophilized RNAse used here (see 21) is identical with the sum of the extinction coefficients for all of the constituent amino acids in RNAse.

Even if our assumption is wrong and there is a difference in the cystine absorption inside RNAse, it seems unlikely that our current observations depend upon only a

simple change in the absorption spectra due to the internal pH being different than pH 5. That is, the RNAse and glutathione values do not differ by a constant factor at the various wavelengths: at 265 nm the $\phi_{1/2\ cys}/f_{cys}$ in RNAse is less than that in glutathione, whereas the reverse is observed at 229 and 280 nm.

We conclude that there are also differences in the photochemical response of histidine "inside" lysozyme in solution. Specifically, from the data of Ferrini and Zito (19), the quantum yield calculated for the loss of histidine for those photons absorbed only by histidine would be approximately 1000. It seems impossible that such a large change in extinction coefficient could arise from incorporation of histidine into a protein. Accordingly, incorporation of an amino acid into a protein changes its photochemical behavior from that for the amino acid in solution or in a tripeptide. Further, the behavior of histidine is greatly different in lysozyme (19), carboxypeptidase A (20), and RNAse.

As anticipated from numerous studies with organic compounds, the yields and the distribution of products vary appreciably with the exciting wavelength (Table III); note that significant histidine destruction is only observed with 229 nm light. Considerable variation in the yields for oxidized glutathione is observed also. As can be seen in column 2 of Table II, the yield for the destruction of half cystine in this compound differs by at least a factor of two for the four irradiation wavelengths studied.

(c) *Photons Absorbed by the Aromatic Residues can Cause the Destruction of the Integrity of Other Residues*[3]

The cystine yields in oxidized glutathione and in RNAse are very similar at 265 and 254 nm where the fraction of the light absorbed by the cystines is relatively

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[3] Dose tried to express the total loss of one-half cystines ($\phi_{1/2\ cys}$) in *all* proteins due to 254 nm irradiation by the following summation (11):

$$\phi_{1/2\ cys} = (\phi_{S-S})(f_{cys}) + (\phi_{\underset{Tyr+Phe}{S-S}})(f_{Tyr+Phe}) + (\phi_{\underset{Try}{S-S}})(f_{Try})$$

where $\phi_{\underset{Cys}{S-S}}$, $\phi_{\underset{Tyr+Phe}{S-S}}$, and $\phi_{\underset{Try}{S-S}}$ are the quantum yields for the loss of half cystines as a result of photons absorbed in constituent cystines, tyrosines + phenylalanines, and tryptophans, respectively, and the f's are the corresponding fractions of the light absorbed by these entities. Even though the disruption of a particular amino acid is known to depend strongly upon its environment (7, 23) he assumed ϕ_{S-S} to be the same in all proteins as in cystine alone in solution and he hoped to find $\phi_{\underset{Tyr+Phe}{S-S}}$ and $\phi_{\underset{Try}{S-S}}$ to be constant also in all the proteins. In making his calculations, Dose assumed ϕ_{S-S} to be 0.12, the value for cystine irradiated alone in solution, even though the proteins were irradiated at pH's different from that for which the cystine data were obtained. However, with these assumptions the values for $\phi_{\underset{Tyr+Phe}{S-S}}$ calculated from the data for RNAse and insulin (0.032 and 0.064, respectively) varied by a factor of two (11). This discrepancy between proteins is not surprising since it is known from phosphorescence studies that energy transfer among the aromatic residues depends critically on the internal environment of individual proteins (9, 24, 25, 26). Further, we have investigated whether a constant set of values would be obtained for RNAse irradiated at different wavelengths

large. However, at 280 nm, where more than 90% of the light is absorbed by tyrosine, $\phi_{1/2\ cys}/f_{cys}$ for RNAse is almost twice that for glutathione which contains no aromatics. At 229 nm the number of the one-half cystine residues destroyed in RNAse is greater than the number of photons absorbed directly by cystine (i.e. $\phi_{1/2\ cys}/f_{cys}$ is greater than one) whereas in oxidized glutathione the respective value is only 0.22. To explain this simply on the basis of changes in the absorption spectrum would require that the extinction coefficient of cystine increase by a factor of six when it is incorporated into RNAse.

Since this seems extremely unlikely it is worthwhile to estimate what fraction of the cystine disruption results from photons absorbed initially in other residues. If the cystine absorption and the photochemical yield for the photons absorbed by cystine are the same as in oxidized glutathione, then at 229 nm the quantum yield for cystine loss from those photons absorbed in the RNAse cystines should be 0.004 (i.e., 0.02 of the 229 nm light is absorbed by RNAse cystine and the $\phi_{1/2\ cys}/f_{cys}$ = 0.22 for glutathione irradiated at 229 nm). Thus, approximately 85% of the value of $\phi_{1/2\ cys}$ = 0.026 (column 4, Table III) must reflect the effects of photons absorbed elsewhere in the molecule.

Carrying out this procedure on the data at 280 nm gives an expected yield of $\phi_{1/2\ cyst}$ = (0.25) (0.06) = 0.015 for those photons absorbed directly in cystines; the remaining yield of 0.009 presumably arises from the 94% of the 280 nm photons absorbed in the aromatics, and if so, the quantum efficiency for half cystine destruction by these photons must be ~0.01 at this wavelength. This low value may imply that the critical interaction is preceded by fairly specific energy transfer (9, 22).

At the other two wavelengths expected yields are obtained as follows:

$$254\ \text{nm}\ \phi_{1/2\ cys} = (0.39)\ (0.33) = 0.13$$

$$265\ \text{nm} \qquad = (0.44)\ (0.14) = 0.062$$

The first value is very close to the 0.12 actually observed, but the latter is significantly larger than the 0.043 produced in RNAse by 265 nm light. Accordingly, we now have underway an investigation of the yields for disruption of the four *individual* cystines in RNAse by the four wavelengths studied here. One objective is to determine if cystine-aromatic interactions can actually stabilize cystines excited

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within the lowest-lying excitation band (since RNAse does not contain tryptophan the last term in the above equation can be deleted). As noted above, at 280 nm using the $\phi_{1/2\ cys}$ = 0.016 we found for RNAse and the $\phi_{\underset{cys}{S-S}}$ = 0.25 obtained in oxidized glutathione, $\phi_{\underset{Tyr+Phe}{S-S}}$ is calculated to be 0.01. But when the same procedure is applied to the data for 254 and 265 nm, negative values are obtained for $\phi_{\underset{Tyr+Phe}{S-S}}$. Obviously, when photons having these three wavelengths are absorbed by aromatics they have different effects on the cystines. Thus, we conclude that while the above equation may be appropriate for a given wavelength and set of environmental conditions, there is no universal set of constant values for these parameters in proteins. This presumably indicates that there are appreciable differences in the interactions between constituent aromatics and cystines in different proteins.

to certain levels in contrast to the enhanced disruption at other wavelengths (e.g., 229 or 280 nm).

(*d*) *Major Mechanism of RNAse Inactivation Persists at Various Wavelengths*

The parallelism between ϕ_E and $\phi_{1/2\ cyst}$ and ϕ_{SH} suggest that some mechanisms persist throughout the range studied. In particular, this suggests that cystine disruption is perhaps the most important mechanism of RNAse inactivation in this wavelength range.

In contrast to this constancy, "reactivation" of RNAse could only be demonstrated following irradiation with 254 nm light but not with the other wavelengths. This reactivation, however, appears to be associated with at most 15% of the total inactivation.

The tyrosine data provide additional evidence that at least one other inactivation mechanism may vary with wavelength. Specifically, no tyrosine is lost at 254 and 265 nm but at 229 nm the number of tyrosines lost per molecule of RNAse inactivated is close to 1.0.

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