THE ACTION OF MONOCHROMATIC ULTRAVIOLET LIGHT ON PROTEINS

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INTRODUCTION

It is desirable to understand the mechanism by which ultraviolet light affects proteins. Such knowledge is not only of interest in its own right but also will shed light on the mechanism by which ultraviolet light affects living systems, and put on a firmer foundation the many action spectra which have been obtained in the past. Finally, this knowledge will be useful in interpreting the action of ionizing radiation on living systems, even though ultraviolet and ionizing radiation are basically different in their effects.

The subject of the action of ultraviolet light on proteins and their constituents has been ably summarized by McLAREN[1]. More recent shorter reviews have been given by ERRERA[2] and by DOTY AND GEIDUSCHEK[3]. The available evidence to date indicates that the quantum yield for the inactivation of proteins, that is — the number of molecules inactivated per absorbed photon, depends on the wavelength. Experiments which show this wavelength-dependence have been carried out for the enzymes pepsin, urease, trypsin (see ref. [1]) and chymotrypsin[4]. Supporting evidence for this conclusion comes from the fact that the pH-dependence of the quantum yield depends on whether monochromatic or heterochromatic radiation is used for the inactivation of pepsin[5] and triosphosphate dehydrogenase[6].

Two other generalizations about the light-inactivation of proteins have been found. First, the quantum yield for inactivation decreases as the molecular weight of the molecule increases[7]. Quantitatively quantum yield is proportional to $1/M$. Second, the quantum yield is proportional to the relative amount of cystine in a molecule[8]. These two relations are not necessarily independent of one another because for the molecules which have been studied photochemically, and for which amino acid analyses are available, the amount of cystine is inversely proportional to the molecular weight[9]. More experimental information is needed before we can decide how these three relations are related to one another. It may be, for example, that large molecules have a small quantum yield because they have little cystine, or alternatively that small molecules have high quantum yields not because they have a large proportion of cystine but simply because they are small.

We have investigated the action of monochromatic ultraviolet light on the proteins trypsin, ribonuclease and aldolase, and the cyclic polypeptide gramicidin.

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We were able to perform the experiments because we had available high-intensity light sources and monochromator. Each of these molecules has special properties which make it suitable for irradiation studies. They are described in detail in the separate sections below.

GENERAL EXPERIMENTAL CONSIDERATIONS

The results obtained from the irradiation of specific enzymes will be discussed below. The following experimental techniques, however, are common to all the experimental results. Monochromatic ultraviolet light was obtained by use of a large water-prism monochromator[10]. The light source was a water-cooled 1000 watt high-pressure mercury lamp. For wavelengths 1850 A and 1943 A we have used an open water-prism monochromator with a 250 watt medium-pressure mercury lamp as the light source. Typical intensities were as follows: at 1850 A, 10^3 ergs/cm^2/min; at 2300 A, $4 \cdot 10^4$ ergs/cm^2/min; at 2650 A, $2 \cdot 10^5$ ergs/cm^2/min; at 3131 A, 10^6 ergs/cm^2/min. We have not investigated in detail the effect of different intensities, but over a factor of five the results of irradiation at low intensities and long times are the same as for high intensities and short times. It has been shown[11] that the reciprocity law holds at 2537 A for the inactivation of pepsin over an intensity range of 10^5. No effect of different concentrations of irradiated material has been observed over the ranges used in these experiments.

The light intensity incident on a sample was measured with a GL-935 photocell which was calibrated against a thermopile and standard lamp combination. The scattered light was negligible at all wavelengths except 2300 A. At this wavelength the scattered intensity amounted to about 10% of the incident intensity. The scattered light was all of much longer wavelength and since the longer wavelengths are much less effective in inactivation than 2300 A, their intensity was simply subtracted from the original reading. At 3131 A the scattered light was very small but consisted of shorter wavelengths. The shorter wavelengths are much more effective than 3131 A in inactivating proteins, so we filtered the incident light through a thin piece of plastic (Mylar). This plastic film transmits no wavelengths shorter than 3050 A. Because of short wavelength scattered radiation, our experimental results at 3023 A may indicate too high a sensitivity.

Irradiation of dry material was carried out in the following way. Five hundredths of a milliliter of a protein solution were pipetted onto $\frac{1}{4} \times \frac{7}{8}$ inch glass cover slips. The solution was evaporated in a vacuum desiccator and the cover slips were then transferred to a vacuum chamber for irradiation. The vacuum chamber was evacuated through a liquid air trap and therefore the water vapor pressure was negligible. Light entered the irradiation chamber through a quartz window. Correction was made for the reflection of light by this window. For irradiations at liquid air temperature the cover slips were placed on a liquid-air-filled container within the vacuum chamber.

Solutions were irradiated in quartz cells 4 cm high with a 1 × 1 cm cross section. They were stirred with a small magnetic stirrer. All irradiations were carried out at room temperature unless otherwise noted.

Some of the irradiated solutions and dry samples absorbed an appreciable fraction of the incident light. In these cases the average incident intensity through the sample was computed by the method given by MOROWITZ[12].

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The absorption spectra of the various protein solutions were obtained with a Beckman spectrophotometer Model DU. Absorption spectra at low wavelengths were obtained by using the radiation from the open water prism monochromator and by use of a vacuum grating-monochromator. Spectra with the samples at liquid air temperature were obtained by drying the material on thin quartz cover slips which were then cooled to liquid air temperature by surrounding metal shields.

GENERAL THEORY

Light, in passing through a system of independent absorbing units, is absorbed according to the equation $I/I_0 = e^{-nsx} = 10^{-\varepsilon cl}$, where I_0 is the incident intensity, I the intensity emerging from a solution of thickness x or l, whose concentration is either n particles/ml or c moles/liter. The absorption coefficient is given by either s or ε. In the former case it has the dimensions of square centimeters. It will be referred to as the absorption cross-section. ε is the molecular extinction coefficient. The two parameters are related by the formula $s = 3.83 \cdot 10^{-21}\,\varepsilon$[13]. The absorption cross-sections for proteins in the ultraviolet region are of the order of 10^{-16} cm^2. We shall occasionally give these cross-sections in units of A^2.

The inactivation of enzymes by ultraviolet light is independent of the intensity and depends only on the product of intensity and time of irradiation[11]. The activity, either enzymic or any other measure, is given by the expression

$$n/n_0 = e^{-\sigma D}. \qquad \text{Eqn. (1)}$$

n/n_0 represents the relative activity after an incident dose of radiation D ergs/cm^2. The coefficient σ has the dimensions of area/energy and will be called the inactivation cross-section. It may be expressed in square centimeters/erg or in square centimeters/incident quantum. The latter unit is the proper one for comparison with absorption cross-sections.

The number of molecules inactivated per absorbed quantum is known as the quantum yield, Φ, and is given by $\Phi = \sigma/s$. If all absorbed quanta are equally effective in producing inactivation, the quantum yield should be independent of wavelength. To a good approximation we may consider that the absorption cross-section of a protein molecule is the sum of the absorption cross-sections, s_i, of the individual absorbing units such as tyrosine, tryptophan, cystine, etc.[14]. Light absorbed by an individual unit of a protein molecule, *e.g.* the i^{th} unit, will have a probability φ_i of causing inactivation with an inactivation cross-section $\sigma_i = \varphi_i s_i$. The inactivation cross-section for the molecule as a whole may then be written as

$$\sigma = \Sigma \sigma_i = \Sigma \varphi_i s_i. \qquad \text{Eqn. (2)}$$

Because the s_i are all different functions of the wavelength it is seen that the quantum yield

$$\Phi = \Sigma \varphi_i s_i / \Sigma s_i \qquad \text{Eqn. (3)}$$

will *not* be independent of the wavelength unless all φ_i are equal and then only if φ_i is independent of wavelength.

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DRY TRYPSIN

We have investigated the inactivation of trypsin at room temperature and at liquid air temperature to see whether the decrease in radiation sensitivity found at 2537 A[15] is the same at other wavelengths. The wavelength range covered was 1850 to 3131 A.

The molecular weight of trypsin has been uncertain. Early values were in the neighborhood of 34,000. Later estimates are about 24,000[16]. Recently NEURATH* has found a minimum molecular weight of about 23,000. His data indicate that trypsin contains about eight half-cystine residues per molecule. We have assayed the enzymic ability of trypsin by its ability to catalyze the breakdown of casein[16]. To simplify the comparison of our data with those of previous workers we have used 34,000 as the molecular weight in the computation of absorption coefficients and quantum yields.

Samples of dry trypsin** for irradiation were prepared by drying 10 micrograms (in 0.0025 N HCl) on rectangular glass cover slips. The absorption spectrum of trypsin at liquid air temperatures was the same as at room temperature. Therefore any effects which are observed at low temperatures are not due to a change in the absorption of radiation but a different mode of energy dissipation.

Results and discussion

The inactivation of trypsin by ultraviolet light follows Eqn. (1). Typical results are shown in Figs. 1 and 2. When the incident dose is such that $n/n_0 = 0.37$ then $\sigma D_{37} = 1$ because $e^{-1} = 0.37$. For example, in Fig. 1 for the inactivation at 2537 A we see

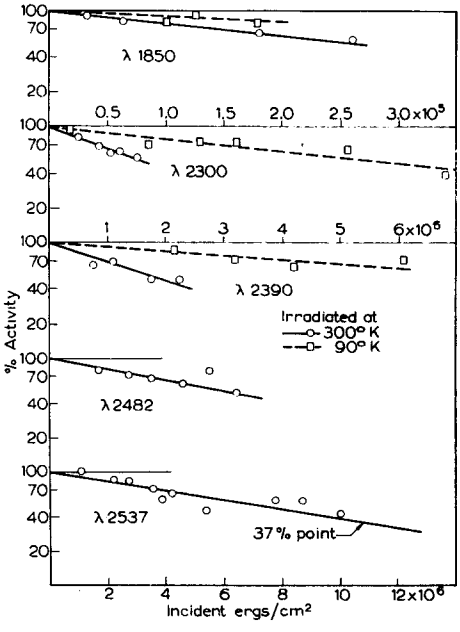

Fig. 1. The relative activity, on a logarithmic scale, of dry trypsin *versus* the incident dose of radiation at particular wavelengths. The samples were irradiated at the indicated temperature.

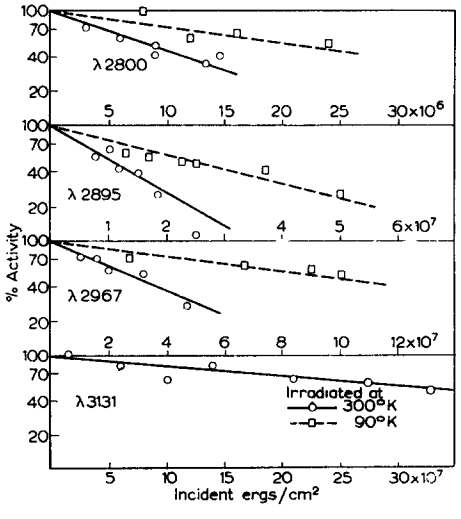

Fig. 2. Activity curves for dry trypsin similar to those shown in Fig. 1.

* We are indebted to H. NEURATH for informing us of these results before they were published.

** 2 × crystallized, salt-free, from Worthington Biochemical Corporation, Freehold, New Jersey.

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that the 37% dose corresponds to $10.8 \cdot 10^6$ ergs/cm^2. This corresponds to a cross-section equaling

$$1/(10.8 \cdot 10^6 \text{ ergs/cm}^2) = 9.3 \cdot 10^{-8} \text{ cm}^2/\text{erg} = 7.2 \cdot 10^{-3} \text{ A}^2/\text{photon}.$$

The data shown indicate that at all wavelengths the inactivation cross-section at 90° K is less than that at 300° K. Fig. 3 shows the action spectrum for the inactivation of dry trypsin as compared to its absorption spectrum. The absorption spectrum shown was obtained on trypsin in solution but the differences between this spectrum and between the spectrum of dry trypsin are negligible. It is easier, however, to obtain proper absolute values for material in solution.

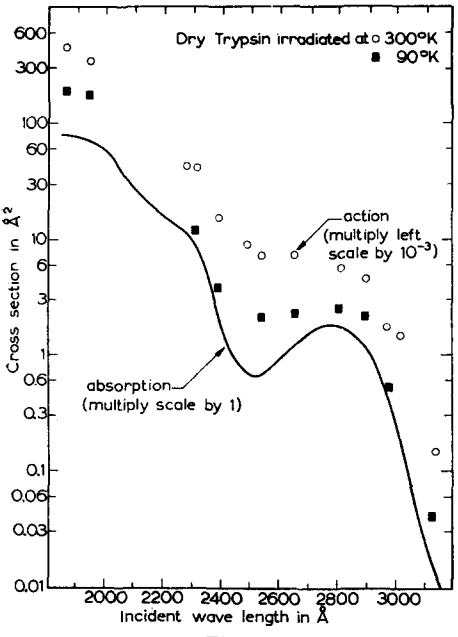

Fig. 3

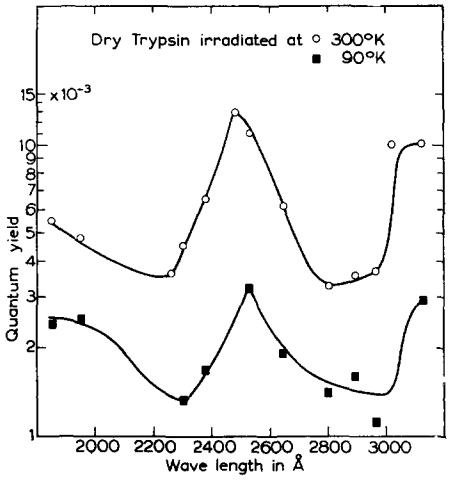

Fig. 4

Fig. 3. The absorption spectrum and the action spectra of dry trypsin irradiated at 300° K and 90° K.

Fig. 4. The quantum yield for the inactivation of dry trypsin versus the wavelength.

The obvious conclusion to be drawn from the data is that the action spectrum is not parallel to the absorption spectrum. The effect of the absorbed light is relatively larger at 2500 A than at 2800 A. An alternative way of indicating the lack of parallelism between the two spectra is to plot the quantum yield as a function of wavelength. Fig. 4 shows the data treated in this fashion. The quantum yield has maxima at 2500 A and at 3100 A for both irradiations at 300° K and 90° K. To a good approximation the two curves of Fig. 4 are parallel to one another. They differ only by a constant factor. Therefore if $\varphi_1(\lambda)$ represents the wavelength-dependence of the quantum yield and $\varphi_2(T)$ represents the temperature-dependence of the quantum yield, then $\Phi(\lambda,T) = \varphi_1(\lambda)\,\varphi_2(T)$. The decrease in sensitivity of proteins at liquid air temperatures has been interpreted as arising from an increase in the fluorescence of proteins at low temperatures[15], and is not related to any change in the absorption spectrum. These data indicate that the energy-transfer mechanisms which are responsible for the increase in fluorescence are of equal efficiency at all wavelengths.

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The absolute value of the quantum yield at 2537 A is in excellent agreement with the value found by GOLDENBERG AND MCLAREN[17] for the inactivation of trypsin in solution*. The shape of the quantum yield *versus* wavelength curve is qualitatively similar to that found by VERBRUGGE and by UBER AND MCLAREN[1], although our data for the irradiation of dry trypsin indicate a bigger difference between the effects of 2357 and 2800 A radiation. The irradiation of trypsin in solution (see below) does not give such a large difference between these two wavelengths.

The action spectra shown in Fig. 3 look much more like the absorption spectrum of cystine than like the absorption spectrum of trypsin. In what follows we shall assume that we may resolve the effect of ultraviolet light on dry trypsin into two components: one component representing photons absorbed by cystine**, the other representing photons absorbed by the remainder of the molecule. Let the subscript c represent cystine and m represent the remainder of the molecule. Because there are four cystine groups per 24,000 molecular weight molecule, Eqn. 2 takes the form:

$$\sigma = \varphi_m s_m + 4 \varphi_c s_c . \qquad \text{Eqn. (4)}$$

It is possible to fit the experimental data for the inactivation of dry trypsin at room temperature by an equation of the above form with the parameters $\varphi_m = 0.002$ and $\varphi_c = 0.07$. In Fig. 5 the curve marked (trypsin-cystine) is a plot of the first term of Eqn. (4) versus the wavelength; the curve marked cystine represents the second term; and the solid curve is the sum of the two components.

It was not possible to carry the calculated values to very low or high wavelengths because of the uncertainty in the absorption coefficients of the individual amino acids at these wavelengths. The agreement between the experimental and the calculated expression is superb and clearly is not fortuitous.

Cystine contributes less than 10% to the absorption coefficient of trypsin and we may replace Σs_i by s_m. Eqn. (3) then takes the form:

$$\Phi = \varphi_m + \varphi_c (s_c/s_m)$$

When s_c/s_m is relatively large, Φ will be large. This is the explanation for the large yield at 2500 and 3100 A.

The large sensitivity of cystine relative to the other components of dry trypsin is shown by the values of $\varphi_c = 0.07$ compared to $\varphi_m = 0.002$ for the rest of the molecule. The fact that the action spectrum may be resolved into these components indicates that the aromatic amino acids (which absorb strongly at longer wavelengths) and the peptide bonds (which absorb strongly below 2200 A) have about equal yields for inactivation. The value for cystine, 0.07, is larger than the value, 0.02, found for the conversion of cystine to cysteine[19].

We may also resolve the action spectrum at 90° K into two components. When this is done we obtain $\varphi_m = 9 \cdot 10^{-4}$ and $\varphi_c = 0.02$. Compared to room temperature the quantum yield for cystine has decreased by a factor of 3.5 while that for the remainder of the molecule has decreased by 2.3. The parallelism between the two action spectra is not exact. However, the numerical figures are sensitive functions

* The values shown in Fig. 4 are for a molecular weight of 34,000. If the value 24,000 is used, the quantum yields would be larger by a factor of 1.4.

** The absorption spectrum of cystine depends on the neighboring amino acids[18]. We have used the values for cystinyldiglycine.

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of the inactivation cross-sections. It would be premature to say that the difference in shape between these two curves is anything but experimental error.

Fig. 5 shows that the mechanism of inactivation at wavelengths 2500 and 2800 A may be quite different. At 2800 A cystine represents one half of the inactivation effect while at 2500 A it represents about 80% of the inactivation effect. We therefore irradiated trypsin in solution with these two wavelengths and then obtained the sedimentation patterns in an ultracentrifuge.

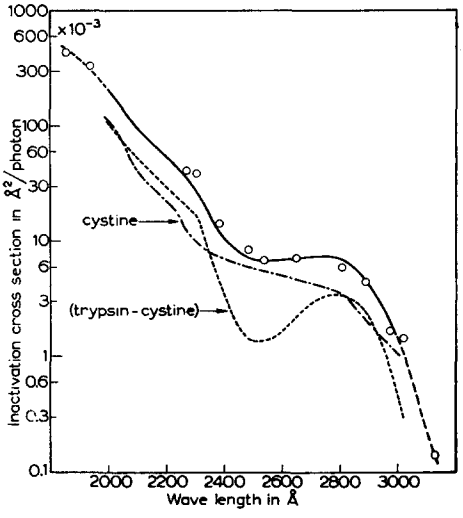

Fig. 5

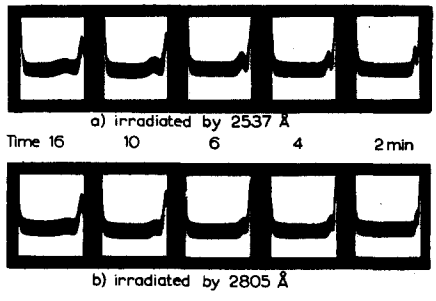

Fig. 6

Fig. 5. The decomposition of the 300° K action spectrum for dry trypsin into a component proportional to the cystine absorption spectrum and a component proportional to the absorption of the molecule without cystine. The solid curve is the sum of the two components and the points represent experimental values.

Fig. 6. Sedimentation patterns, at the indicated times, of trypsin irradiated to 15% relative activity with (a) 2537 A and (b) 2805 A radiation. The small rapidly moving peak arises from the irradiation. The large slow peak is native trypsin. The samples were spun at pH 2.1, $\mu = 0.1$; 200,000 × g; bar angle 45°.

WET TRYPSIN

Trypsin was irradiated at a pH of 2.1 and a salt concentration of 0.1 M NaCl. The trypsin concentration was 5 mg/ml. For these very absorbing solutions the quantum yield was calculated by the method outlined by McLAREN[1]. The quantum yields obtained under these conditions are slightly higher than those obtained for dry trypsin and the variation in the quantum yield from one wavelength to another is not as pronounced. We found quantum yields at 2805 A of $0.8 \cdot 10^{-2}$ and at 2537 A of $1.6 \cdot 10^{-2}$. If, as before, we separate the effect into cystine and noncystine components, we obtain $\varphi_m = 0.005$ and $\varphi_c = 0.10$. From these data it looks as if trypsin in solution has an increased sensitivity because of an increase in the aromatic amino acid sensitivity. The sensitivity of the cystine component is about the same.

The sedimentation patterns of unirradiated and irradiated trypsin were observed in a Spinco Model E analytical centrifuge at centrifugal fields of 200,000 × g. Fig. 6 shows a comparison of a sedimentation diagram of trypsin irradiated at 2805 A and at 2537 A. It is seen that the effect of the radiation in both cases is to produce a high-velocity sedimenting component. The sedimentation constant of this large component is about 20 s compared to the value observed for native trypsin of 2 s.

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The high velocity peak does not represent homogeneous material. It diffuses out quite rapidly. For a given loss in enzymic activity the peak is more prominent and more homogeneous for 2537 A radiation than for 2805 A. For both wavelengths, the lower the enzymic activity the higher the magnitude of this second peak. These results show that 2537 and 2805 A radiations inactivate the enzymic activity of trypsin by non-identical mechanisms.

McLAREN[1] has observed an increase in the amount of high- and low-velocity components after irradiation of urease. But the production of large components does not seem as specific as is observed for trypsin. The production of large molecular weight aggregates upon irradiation of bovine serum albumin has been observed by RIDEAL AND ROBERTS[20] from the osmotic pressure change after irradiation. The authors interpret this result as arising from a break in the peptide bond and a re-aggregation of the free radicals so formed with native molecules. An alternative explanation is that the free radicals which are produced are in part S-groups which may aggregate with native molecules.

GRAMICIDIN

Gramicidin is a cyclic polypeptide. Its properties have been reviewed recently[21]. It has a molecular weight of 8,700 and contains, as its major absorbing constituent, tryptophan. It has no tyrosine or cystine*. The antibiotic activity of gramicidin was assayed by its ability to inhibit the growth of *Staph. aureus*. Each sample was assayed in duplicate at three different dilutions. There were, as is seen in the data presented below, three irradiated points at each wavelength. The relative activity of the irradiated gramicidin was obtained by comparing its antibiotic effect with that given by a standard curve prepared anew for each wavelength. The standard curve was constructed by plotting the growth of *S. aureus* against the amount of gramicidin per growth tube of 20 ml. Six concentrations of gramicidin were used, each done in quadruplicate, up to a maximum of 0.16 μg/tube.

Gramicidin for irradiation was dissolved in ethyl alcohol to a concentration of 50 μg/ml. Five hundredths ml samples were taken for assay and were diluted as indicated above in a solution made up of propylene glycol (44 ml) alcohol (12 ml) and water (to make 100 ml). The wavelength range covered was 2300 to 3023 A.

Results

Typical experimental results are shown in Fig. 7. These data are not as accurate as those obtained for trypsin but nevertheless they indicate that within experimental error Eqn. (1) is obeyed. The inactivation is exponential. Much higher doses of radiation are needed to inactivate gramicidin than to inactivate trypsin. Fig. 8 shows the action and absorption spectra of gramicidin and also the quantum yield as a function of wavelength. The absorption and action spectrum are approximately parallel to one another and the quantum yield is constant with wavelength. The slight increase in quantum yield as the wavelength decreases is close to the experimental error and we are justified in saying that from 2300 to 3000 A the quantum yield for inactivating gramicidin is constant. Photons of different wavelengths absorbed

* We are grateful to the Wallerstein Laboratories for supplying the crystalline gramicidin used in this work.

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by tryptophan are equally effective in inactivation. If the slight rise in quantum yield at low wavelengths is real, it may come from absorption by the peptide bonds or energy absorbed in the lower wavelength absorption band of tryptophan.

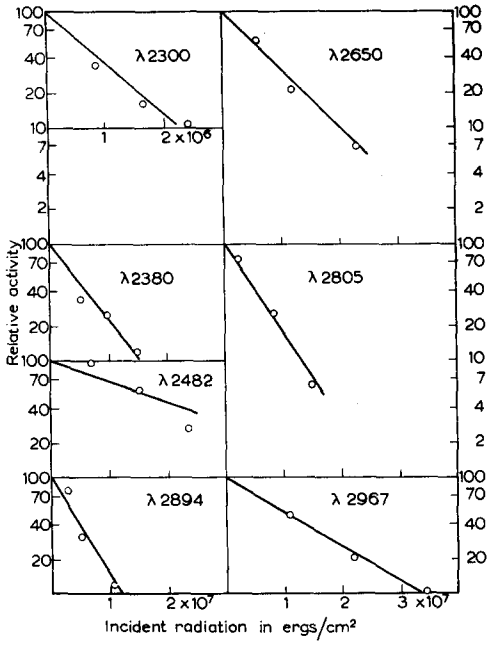

Fig. 7. Inactivation curves for gramicidin at several wavelengths.

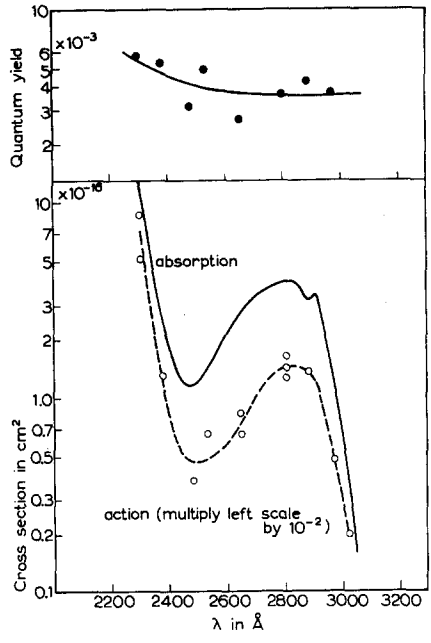

Fig. 8. A comparison of the action and absorption spectrum for gramicidin. The upper graph shows the variation of quantum yield with wavelength.

RIBONUCLEASE

Ribonuclease has a molecular weight of about 15,000[22]. It contains 7 tyrosines and 8 half-cystines as its major absorbers at long wavelengths and has no tryptophan[22]. The spectrum may be changed by going from acid to alkaline pH. The change arises from the ionization of the –OH group of tyrosine[14]. The change in absorption by cystine is much smaller. At 2537 A about half the absorption in acid solution is due to cystine. It is known from enzymic degradation experiments that not all of the ribonuclease molecule is essential for its enzymic activity[23, 24]. However, splitting S–S bonds in the molecule does inactivate it.

Dry RNA-ase* was irradiated at surface densities of 5 μg/cm^2. Irradiations in solution covered a concentration range of 20 to 4 μg/ml. Acid solutions were 0.0025 N HCl, basic solutions were 0.01 N NaOH. Irradiations at 2300 A, in both acid and alkali, were carried out with oxygenated and nitrogenated solutions to test for the possible existence of any indirect effects at this and higher wavelengths. No difference in the two cases was observed.

After irradiation the samples were assayed for enzymic activity by the method outlined by McLAREN *et al.*[7].

* Crystalline, obtained from Armour and Co., Chicago, Illinois.

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Results

Typical experimental results are shown in Figs. 9 and 10. The inactivation is exponential, and at all wavelengths except 2300 and 2890 A the cross-section in alkali is larger than in acid solution. These experimental results are summarized in Fig. 11. The points represent inactivation cross-sections and the curves represent the absorption spectra and indicate clearly the change in spectrum arising from the change in pH. At the alkaline pH the tyrosine groups are not completely ionized.

The sensitivity near 2600 A of RNA-ase dried from acid solution is about the same as for the enzyme in solution. At liquid air temperature the cross-section is less than at room temperature by a factor of 2. This factor is somewhat smaller than factor of three which has been found for other proteins[25]. Two generalizations may

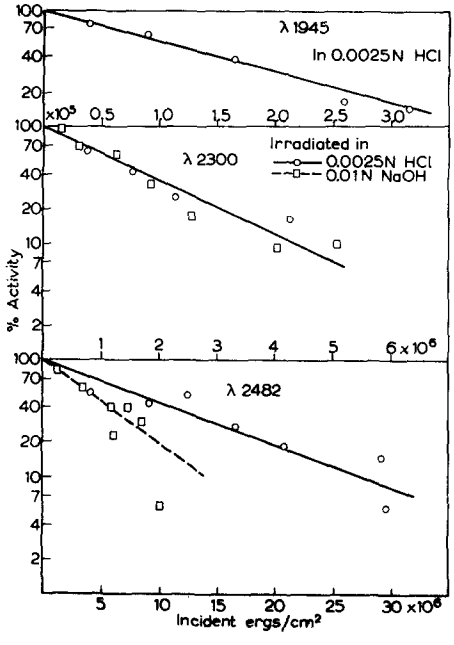

Fig. 9. Inactivation curves for RNA-ase in acid and alkaline solution.

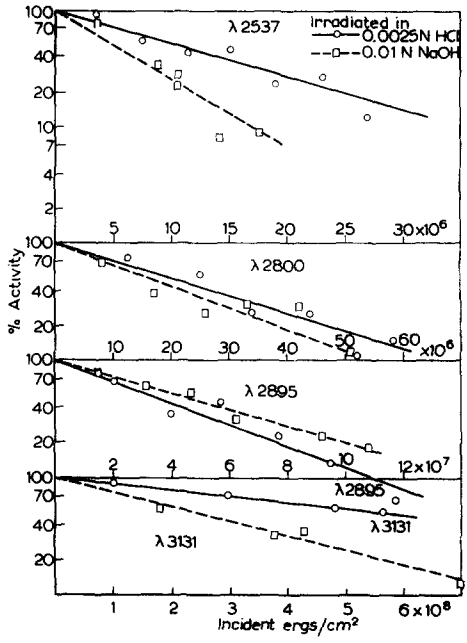

Fig. 10. Inactivation curves for RNA-ase in acid and alkaline solution.

be made from the data presented in Fig. 11. A large change in the absorption spectrum produces a small change in the action spectrum, and the cross-sections at 2300 A and 2800 A, where the absorption cross-sections are the same, are equal. This last point is best exemplified by the curve of quantum yield *versus* wavelength shown in Fig. 12. The absolute value of the quantum yield for the inactivation of RNA-ase in acid solution at 2537 A agrees well with the value 0.03 found by others[7].

The quantum yield is a maximum at 2500 A and it is higher at 3131 A than at 3000 A. Because the wavelength dependence is similar to that obtained for trypsin, it is tempting to analyze the RNA-ase data in the same way as was done for trypsin. However the action spectrum calculated from the sum of cystine and the rest of the molecule is too low by as much as a factor of 2 for wavelengths below 2600 A if theory is fitted to the data for longer wavelengths. (This is a small error compared

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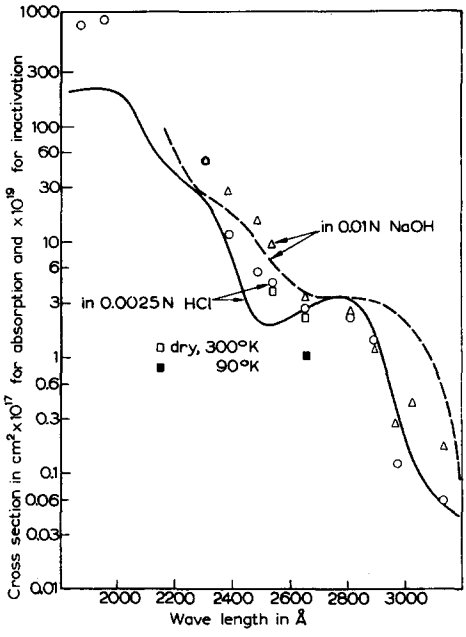

Fig. 11

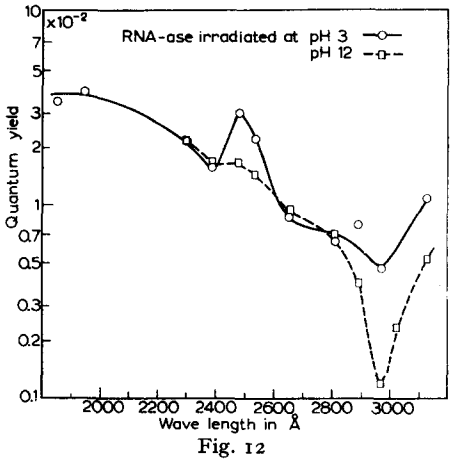

Fig. 12

Fig. 11. A comparison of the action and absorption spectra of RNA-ase in acid and alkali. The curves represent the absorption spectra. Some data for the inactivation of dry enzyme are shown.

Fig. 12. The variation of RNA-ase quantum yield with wavelength for acid and alkaline medium.

to the change in cross-section by a factor 10^4 over the wavelength range studied.) Part of the discrepancy may be the result of improper knowledge of the appropriate cystine absorption spectrum because the spectrum depends on the neighboring groups[18]. Alternative explanations would be that more than two components are necessary, energy absorbed in tyrosine may be transferred to cystine, or the quantum yield per amino acid is not independent of wavelength. Evidence for the last two explanations is furnished by the work of SHORE AND PARDEE[26] which indicates a decreasing fluorescent yield toward lower wavelengths. Moreover we have evidence that the tyrosine quantum yield is wavelength-dependent. If we assume that the difference between the action spectra in acid and alkali arises only from a change in the absorption spectrum of tyrosine and (perhaps not justifiably) that the quantum yields for affecting tyrosine and cystine are independent of pH then we see from Eqn. (2) that the change in σ with pH is proportional to the change in s. Fig. 13 shows these data. The individual points on the straight line arise from different wavelengths since each wavelength corresponds to a given change in absorption cross-section. The result obtained for wavelengths below 2800 A is different from that obtained for wavelengths above 2800 A. For the shorter wavelengths the quantum yield for inactivation by absorption in tyrosine (represented by slope of the line) is about 10^{-2}. For longer wavelengths it is 10^{-3}.

ALDOLASE

Aldolase is a protein of molecular weight 140,000. It has 1% half-cystine, 1.2% tryptophan and 5.2% tyrosine residues[27]. The quantum yield for inactivation at 2537 has been reported[28] as about $2 \cdot 10^{-3}$. The low quantum yield is in accord with

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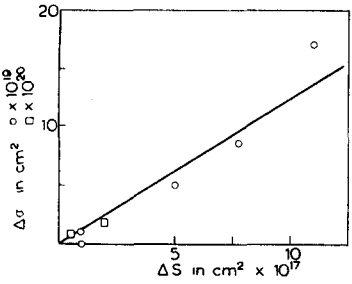

Fig. 13. The change in inactivation cross-section, $\Delta\sigma$, with change in absorption cross-section (obtained by changing the pH). The circles represent different wavelengths of 2800 A and less, the squares represent longer wavelengths. The vertical coordinate is different for the two wavelength ranges.

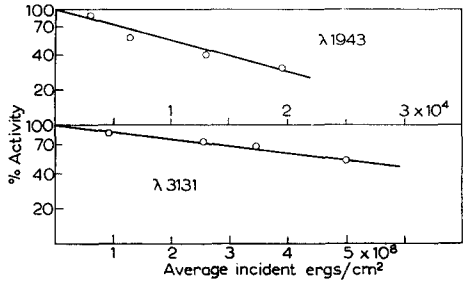

Fig. 14. Inactivation curves for aldolase assayed at 38° C.

its high molecular weight and its low cystine concentration. Most of the crystalline aldolase used in this investigation was supplied through the courtesy of Dr. MELVIN SIMPSON of the Department of Biochemistry in Yale University. Another sample, obtained from the Worthington Biochemical Corp. gave similar results.

Irradiations were carried out in phosphate buffer at pH 7.4. Most of the assays were carried out by the procedure described by SIBLEY AND LEHNINGER[29] in which the split hexosediphosphate is determined by a colorimetric reaction using dinitrophenylhydrazine. These assays were carried out at a temperature of 38° C in a buffer of tris-(hydroxymethyl)-aminomethane. Other assays were performed in this buffer at a temperature of 28° C (see below). Because the results obtained using these assays differed from those obtained by LABEYRIE AND SUGAR[28] we also used their assay in which the activity of aldolase is obtained by measuring, in a Beckman Spectrophotometer, the rate of reduction of DPN in the presence of excess of triosphosphate dehydrogenase. The temperature for this assay was 23° C. The aldolase concentration during irradiation was 40 μg/ml. One-tenth ml samples were removed from the irradiation cell for assay.

Typical experimental data are shown in Fig. 14 and 15. The results are summarized in Fig. 16, which compares the absorption and action spectrum for inactivation of aldolase. Two important facts stand out. First, the inactivation cross-section depends on the method of assay and second, the action spectrum has the same shape as the absorption spectrum except at long wavelengths.

The difference between aldolase assayed at 38° and 28° C arises because irradiated aldolase consists of at least 3 components. One component is enzymically inactive, the other is enzymically active but is very temperature sensitive and inactivated rapidly at a temperature of 38° C, and the third is unaltered aldolase. We have made no detailed study of the rapid heat inactivation of the labile component of irradiated aldolase except to determine that it is over in five minutes. Because the normal assay time at 38° C was 30 minutes, the heat-labile component contributes little to the measured activity. In the spectral region where trypsin and RNA-ase showed large quantum yield changes, aldolase has a constant quantum yield. Our most accurate data, obtained with the 28° C assay, give the values shown in Table I.

The data obtained on aldolase assayed at 38° C are more extensive but less accurate because of the large post-heat effect on irradiated material. For the wave-

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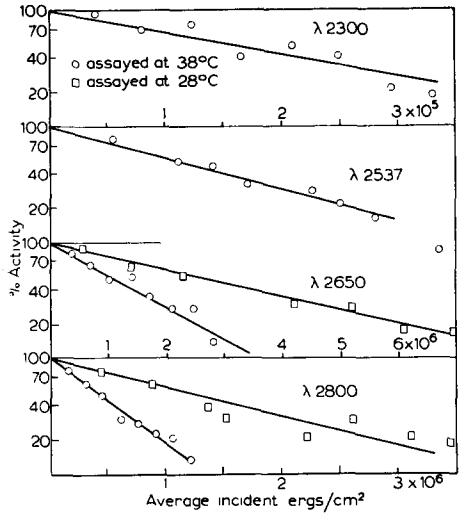

Fig. 15

Fig. 15. Inactivation curves for aldolase assayed at 38° C and 28° C.

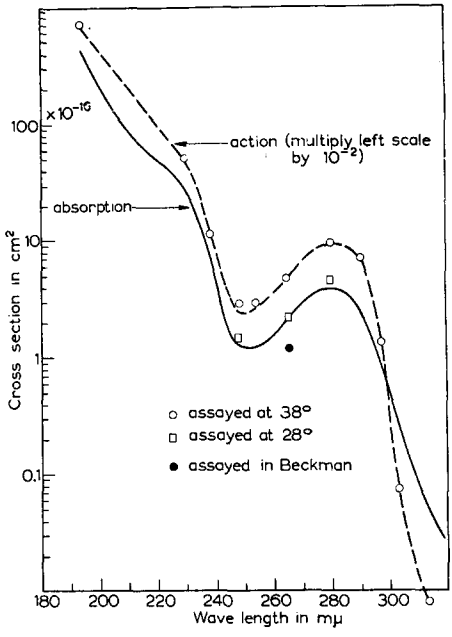

Fig. 16

Fig. 16. A comparison of the action and absorption spectrum of aldolase with different assay conditions.

TABLE I

THE QUANTUM YIELD FOR THE INACTIVATION OF ALDOLASE WHEN ASSAYED AT 28° C

Wavelength	*Quantum yield*
2482 A	$1.10 \cdot 10^{-2}$
2650	1.00
2805	1.16

lengths used the absorption cross-section changes by a factor of 10^4 while the inactivation cross-section increases by $7 \cdot 10^4$. The quantum yield is not constant with wavelength but the variations in it are small compared to the overall change in absorption. In the range 2800 to 1943 A the quantum yield decreases from $2.6 \cdot 10^{-2}$ to $1.6 \cdot 10^{-2}$. The change may be real but it may also arise from errors in the absorption spectrum because any slight impurity will affect the absorption spectrum profoundly at low wavelengths and so alter the calculated values of the quantum yield. The rapid decrease in quantum yield from 2800 A to 3131 A ($2.6 \cdot 10^{-2}$ to $1.8 \cdot 10^{-3}$) is similar to that found for ribonuclease but the increase in yield at 3131 A found for the latter and trypsin is not observed for aldolase. We again note that slight impurities will erroneously raise the absorption cross-section and cause the calculated quantum yield to be too low. The quantum yield found by the method of LABEYRIE AND SUGAR was $5.5 \cdot 10^{-3}$ compared to their value of $2 \cdot 10^{-3}$.

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GENERAL DISCUSSION AND CONCLUSIONS

The chance that an absorbed quantum will inactivate a protein molecule depends not only on the internal organization of the molecule but also on external conditions such as pH and temperature. It is remarkable that any regular correlations are found between the quantum yield and other gross molecular properties such as molecular weight and relative cystine concentration. The average proportionality between Φ and $1/M$ and % cystine assumes the existence of a structure common to all proteins and the equality of all S–S bonds. In extreme environmental conditions, such as the 38° C assay of aldolase, the detailed molecular structure is important and the average correlations may break down. The work reported here on the action spectra for inactivation of aldolase, gramicidin, RNA-ase in acid and alkali, and trypsin at 300° K and 90° K leads to another generalization. Molecules with a high cystine concentration have action spectra which are not parallel to their absorption spectra. The action spectra for trypsin and RNA-ase show a large cystine component, but those for aldolase and gramicidin which have little or no cystine show no such characteristic. The cystine component shows up most where its absorption is relatively high compared to the aromatic amino acids such as at 2500 and 3100 A. The fact that different wavelengths inactivate by different mechanisms is shown by the different sedimentation patterns of trypsin inactivated by 2537 and 2805 A radiation. The ultimate fate of all absorbed photons is not the same.

The curves shown in Fig. 5 indicate that at 1900 A half the effect of light on dry trypsin is on cystine and the other half on the remainder of the molecule. At this wavelength the 220 peptide bonds contribute about 70% of the absorption (see a similar calculation for chymotrypsin[30]). Let φ_p represent the quantum yield for inactivation when a photon is absorbed in a peptide bond. Use of the data of Fig. 3 in Eqn. (2) yields:

$$0.40/2 = \varphi_p \times 75 \times 0.7 + (2 \cdot 10^{-3}) \times 75 \times 0.3,$$

if we assume that the value $2 \cdot 10^{-3}$ for the quantum yield for aromatic amino acids found at long wavelengths holds at short wavelengths. The solution of the above equation is $\varphi_p = 3 \cdot 10^{-3}$. This is of the same order as the quantum yield for splitting the CONH bond in model compounds[31]. The enzymes RNA-ase and aldolase yield φ_p which are within a factor of two of the value for trypsin. Cystine with a quantum yield near 0.05 is by far the most sensitive amino acid.

The unique role of S–S bonds in protein inactivation probably occurs because most of them bind polypeptide chains together. Their breakage may lead to a complete change in form of a molecule with subsequent denaturation and a loss of enzymic activity. The unfolding following S–S bond rupture may be the same as the further unfolding of monolayers as indicated by KAPLAN AND FRASER[32] in their work on the irradiation of monomolecular films by 2537 A radiation. Part of the correlation between quantum yield and cystine content may arise from the relation between cystine content and molecular weight or alternatively from the fact that energy may be transferred to cystine from the other absorbing regions of the molecule. For proteins in solution the large sensitivity of S–S bonds is not sufficient to account for the high quantum yield at wavelength 2800 A.

The results on RNA-ase and aldolase inactivation indicate that the quantum

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yield per amino acid may vary with wavelength. This may be a property of tyrosine because gramicidin (with no tyrosine) shows no such effect. Many questions about the ultraviolet light inactivation of proteins remain unanswered.

SUMMARY

The ultraviolet inactivation spectra are reported for dry trypsin at 300° K and 90° K, gramicidin, RNA-ase in acid and alkali, and aldolase. Trypsin and RNA-ase, which have relatively large amounts of cystine, have action spectra which differ markedly from their absorption spectra. Their action spectra show a large component which is similar to the absorption spectrum of cystine.

The action spectrum for trypsin at 90° K is parallel to, but less than, that at room temperature. The inactivation efficiency of photons absorbed in cystine has been estimated to be 0.05 compared to $2 \cdot 10^{-3}$ for the other amino acids and the peptide bond. The existence of several inactivation mechanisms is shown by the difference in sedimentation patterns of trypsin inactivated at 2537 and 2805 A.

Gramicidin has a constant quantum yield of about $4 \cdot 10^{-3}$.

A large change in the spectrum of RNA-ase, produced by partial ionization of tyrosine in alkali, produced only a small change in the action spectrum. The data indicate that the quantum efficiency for tyrosine is not constant but decreases toward long wavelengths.

The action spectrum for aldolase is parallel to the absorption spectrum except for wavelengths above 2800 A where the RNA-ase data indicate a decrease in the efficiency of photons absorbed in tyrosine. The absolute value of the quantum yield for aldolase depends on the environmental conditions after irradiation.

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