PHOTOLYSIS ACCOMPANYING PEPTIDE ABSORPTION IN PROTEINS

ELECTROPHORESIS AND OPTICAL ROTARY DISPERSION STUDIES

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ABSTRACT Exposure of proteins and polypeptides to ultraviolet radiation below 240 nm produces peptide cleavage which may or may not be accompanied by observable changes in conformation and optical rotary dispersion (ORD) properties, depending on the stability of the secondary and tertiary structure of the macromolecule under the experimental conditions. Helical and coiled forms of poly-L-glutamic acid undergo degradation at similar rates but only the helical form shows a significant change in rotatory properties. The helical form of poly-L-lysine, but neither the coiled nor β forms, shows a change in $[\alpha]_{233}$ on irradiation at 233 nm. β-Lactoglobulin shows essentially no change in $[\alpha]_{233}$ on irradiation in either dilute salt solution or 4 M urea at room temperature; however, in 4 M urea at 56°C a large change occurs. A model is developed which shows that studies of the effect of radiation on ORD properties may be useful in providing information on possible intermediate steps in protein denaturation. The method is illustrated with results on bovine plasma albumin. A quantum yield, 4.3×10^{-3} moles/einstein, was obtained for peptide cleavage in this protein at 225 nm. These studies, based on gel electrophoresis, also showed that the fragments produced are essentially random, suggesting that transfer of energy from aromatic residues is not an important contributor to the peptide photolysis. Possible errors which could arise in ORD and other studies involving intense ultraviolet radiation are considered.

INTRODUCTION

In spite of much research on the photochemical inactivation and denaturation of proteins, little direct evidence has been obtained on peptide cleavage per se (McLaren and Shugar, 1964). Most ultraviolet radiation studies on proteins have been conducted at 254 nm where photolysis of peptide bonds is quite small relative to aromatic and disulfide bond photolysis. Thus, Shugar and coworkers (1959) concluded that the polypeptide protamine, which is devoid of disulfide or aromatic groups, is photolyzed on irradiation at wavelengths below 240 nm but not at 254 nm.

Recently, on the basis of work with small model compounds, it has been proposed that the primary reaction of peptide bonds on photolysis involves bond cleavage with the formation of an amide and an α-keto group (Maybeck and Maybeck, 1967; Maybeck and Windle, 1969).

The authors observed by chance that significant alterations in ORD behavior can occur in some proteins and polyamino acids on prolonged exposure to the radiation in a modern spectropolarimeter. In a short communication (Wilson and Foster, 1970) it was pointed out that changes in ORD were greatest for polyamino acids in the α-helical conformation and for proteins having a relatively high helix content (bovine plasma albumin, myoglobin). Furthermore, with these proteins the effect was greatly accentuated under semidenaturing conditions. Proteins of low helix content (cytochrome *c*, β-lactoglobulin) were reported to give essentially no effect. It was also shown that the effect is greatest in both proteins and in poly-L-glutamic acid at 225 nm and appears to be associated primarily with n-π^* absorption. The present paper presents some further results on polyamino acids which support the conclusion that fragmentation takes place on irradiation independent of the secondary conformation but that observable changes in ORD occur only for the α-helical structure. It is further shown that while β-lactoglobulin shows no observable change in ORD on irradiation at room temperature, it does show a substantial change in $[\alpha]_{233}$ on irradiation in 4 M urea at 56°C. A model is developed to describe the effect of radiation under denaturing conditions. Results of the rradiation of bovine plasma albumin as a function of temperature and in guanidine hydrochloride (G·HCl) of varying concentrations are presented and analyzed in terms of this model. A quantum yield for the photolytic destruction of the peptide chain of bovine plasma albumin is determined by utilizing sodium dodecyl sulfate gel electrophoresis to determine unfragmented reactant as a function of time of irradiation. These experiments also demonstrate an essentially random cleavage on exposure suggesting that energy transfer from aromatic chromophores is probably not an important contributor to the fragmentation process.

EXPERIMENTAL

Materials

Fraction V bovine plasma albumin (BPA) from Armour Pharmaceutical Co. (Chicago, Ill., lot No. D27309) was purified and iodoacetamide blocked by a previously published procedure (Wilson and Foster, 1971). Other proteins used without further purification were: β-lactoglobulin, 3 × crystallized (Pentex Biochemical, Kankakee, Ill., lot No. 28); chymotrypsinogen A (beef pancreas), 6 × crystallized (Mann Research Labs, Inc., New York, lot No. V1177); cytochrome *c* (horse heart), lyophilized powder (Mann Research Labs, Inc., lot No. V1597). Sodium dodecyl sulfate (SDS) was purchased from MC & B Manufacturing Chemists (Norwood, Ohio). Other chemicals for gel preparation were purchased from Eastman Organic Chemicals Div. (Eastman Kodak Co., Rochester, N. Y.). Coomassie brilliant blue R250 was obtained from Colab Laboratories, Inc. (Glenwood, Ill.). Iodoacetamide from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) was recrystallized twice from water before use. Mann

"ultrapure" urea and G·HCl from Heico, Inc. (Delaware Water Gap, Pa.) were used without further purification. Polyamino acids have been previously described (Wilson and Foster, 1970). Other chemicals were of the highest purity commercially available.

Irradiations

Solutions were irradiated either in a Bausch & Lomb instrument (Bausch & Lomb, Inc., Rochester, N.Y.) or in the Cary 60 spectropolarimeter (Cary Instruments, Monrovia, Calif.), and lamp intensities were determined using previously described cells and procedures (Wilson and Foster, 1970). The Bausch & Lomb instrument consisted of a Bausch & Lomb standard ultraviolet grating (200–400 nm) with the lamp compartment and power supply modified to accept a 250 w Osram mercury-xenon lamp. The slit widths were adjusted to give approximately a 10 nm spectral bandwidth for the Bausch & Lomb monochromator and a 1.5 nm bandwidth for the spectropolarimeter unless otherwise indicated. The intensities for the spectropolarimeter (quanta per second per milliliters) were approximately 10 times lower than those for the monochromator at 225 nm. Since the jacketed cell had only about 0.1 the volume of the 22 mm cell used in the monochromator, however, this means that the actual intensity from the spectropolarimeter (quanta per second) was nearly 100 times less than from the monochromator. For this reason the monochromator was used in viscosity and gel electrophoresis experiments where a more highly photolyzed sample was desired in reasonably short time periods.

Thermal Denaturation

A stock solution (0.005% in 0.1 M NaCl, pH = 5.5) of BPA was prepared. At each desired temperature a fresh sample of this solution was placed in the 20 mm jacketed cell, equilibrated for 15–20 min, and scanned from 350 to 220 nm in the spectropolarimeter. The sample was then exposed at 225 nm for 60 min, removed into a syringe for stirring, replaced in the cell, and rescanned.

G·HCl Denaturation

Into a 10 ml volumetric flask the correct volume of a G·HCl stock solution in water was measured and diluted to approximately 9 ml with water. 1 hr before the ORD curve was to be measured BPA was added from a stock BPA solution (0.500 ml of 0.100% BPA solution) and the solution was diluted to the mark with water. The samples were scanned from 350 to 220 nm at 25.0°C using the 20 mm jacketed cell, and exposed for 60 min at 225 nm. After exposure, the samples were stirred and rescanned as described above for the thermal denaturation experiments.

SDS–β-Mercaptoethanol (β-ME) Gel Electrophoresis

The gel procedure involved a slight modification (Wilson and Foster, 1971) of the method given by Weber and Osborn (1969). Since the gels were quantitatively scanned for the amount of protein present, great care was taken in staining and destaining. The gels were stained for 12–15 hr with the recommended Coomassie blue stain, and destained electrophoretically twice for 30 min each time with fresh destaining solution (7.5% acetic acid, 5% methanol in water). After destaining the gels were stored in destaining solution for at least 2 days and then scanned at 500 nm in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a Heath recorder (Heath/Schlumberger Scientific Instruments

Div., Heath Co., Benton Harbor, Mich.). Since 500 nm is not at an absorption maximum for Coomassie, the background is reduced. The Coomassie background seems to remain fairly constant on the gels from protein concentration 0–100 μg and evidently results from the dye binding to the gel.

RESULTS AND DISCUSSION

Irradiation of Polyamino Acids in the Peptide Absorption Region

In a previous communication (Wilson and Foster, 1970) we have reported that $[\alpha]_{233}$ for helical polyamino acids changes appreciably on exposure in the Cary 60 spectropolarimeter at wavelengths in the peptide absorption region. On the other hand, the β and coiled secondary structures do not change their ORD patterns on exposure at several wavelengths in this region. In Fig. 1 $\Delta[\alpha]_{233}$ values ($[\alpha]_{233}$ for the exposed sample minus $[\alpha]_{233}$ for the unexposed sample) are shown for polylysine exposed in the helical, β, and coiled secondary structures (Davidson and Fasman, 1967) with continuous recording of optical rotation at 233 nm.

These results indicate that the helical structure behaves, on exposure, quite differently than the coiled and β forms. This could be because of a difference in rate of reaction on exposure or simply to a difference in ORD behavior of the different secondary structures. To decide which of these alternatives is correct, polyglutamic acid was exposed in the helical and coiled forms at 225 nm in the Bausch & Lomb instrument. Aliquots from both exposed and unexposed helical and coiled forms were diluted to the standard ORD concentration and their pH was adjusted into

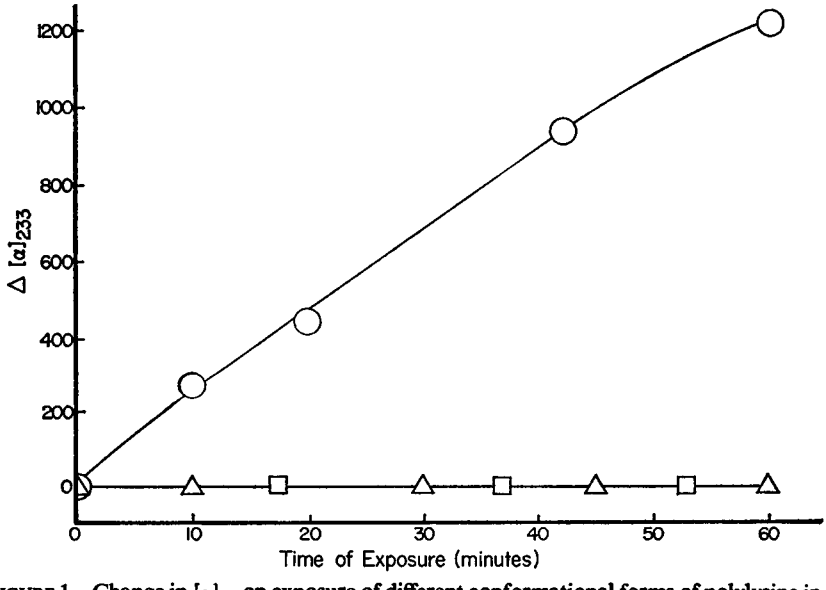

FIGURE 1 Change in $[\alpha]_{233}$ on exposure of different conformational forms of polylysine in the Cary 60 spectropolarimeter at 25°C. Helical form, ○; β structure, △; and coiled form, □.

the helical region (Wilson and Foster, 1970). Quite similar values were obtained for $\Delta[\alpha]_{233}$, namely 227 for the helical form and 212 for the coiled form. The small difference is easily explained by errors in pH adjustment, dilution, and lamp fluctuations during exposure, and it can be concluded that there is no major difference between the rate of photolysis of helical and coiled polyglutamic acid.

The difference in observed ORD behavior between coiled and helical structures on exposure is probably due to the fact that when a helix is cleaved the ORD changes because of end effects. Any end effects in the coil should be small and also there is probably very little unfolding in the coiled form after cleavage. Failure to observe any change in rotation with the β structure is probably due to the large number of hydrogen bonds that must be broken for disassociation of the chains (Davidson and Fasman, 1967).

Irradiation of Proteins in the Peptide Absorption Region

With proteins such as BPA and myoglobin that contain considerable amounts of helix, the ORD curves are dominated by the peptide conformation and cleavages will cause a slight unwinding of the helix and an immediate ORD change (Wilson and Foster, 1970). With other proteins such as β-lactoglobulin and cytochrome *c* whose ORD behavior is largely determined by some other secondary structure or by other chromophores such as aromatic side chains, ORD changes will only be obtained when a cleavage alters the conformational stability of the protein enough to cause it to unfold, giving a change in the environment of the groups contributing to the ORD of the protein.

β-Lactoglobulin, for example, is a protein which gives a trough near 233 nm and seems to contain considerable β structure (Timasheff et al., 1966; Townend et al., 1967). This protein was studied at pH slightly below 3.0 to prevent effects due to aggregation (Pace and Tanford, 1968). On exposure at 233 nm at 25°C in 0.1 M NaCl-HCl only very small changes in $[\alpha]_{233}$ were found. In 4 M urea and at low pH the protein gave no detectable change in optical rotation on exposure with continuous recording at 233 nm at 25°C. On exposure at 45°C a barely detectable change in $[\alpha]_{233}$ was observed, while heating at 56°C gave quite a large change in $[\alpha]_{233}$ on exposure, as shown in Fig. 2. Exposure of β-lactoglobulin at 65°C in 4 M urea gave smaller changes in $[\alpha]_{233}$ than at 56°C so that the maximum change in $[\alpha]_{233}$ occurred near 56°C.

Cytochrome *c* gives an ORD trough at 232 nm but seems, from X-ray data, to contain little regular secondary structure (Beychok, 1968). Exposing this protein at a number of wavelengths gave only small changes in $[\alpha]_{233}$. Also the magnitude of $\Delta[\alpha]_{232}$ did not greatly change with temperature. These results indicate that both conformational stability and secondary structure are important factors in determining the magnitude of $\Delta[\alpha]$ on exposure of a protein in the peptide absorption region. When the ORD curve of a protein contains large contributions due to pep-

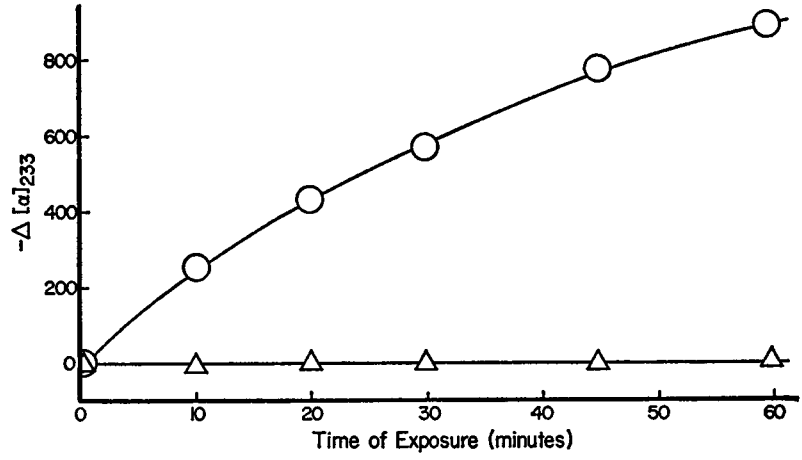

FIGURE 2 Change in $[\alpha]_{233}$ on exposure of β-lactoglobulin in the Cary 60 spectropolarimeter at 25°C, △, and 56°C, ○, in 4 M urea, pH approximately 3.0.

tide groups in a helical conformation $\Delta[\alpha]$ will reflect small changes in secondary structure on exposure in addition to changes due to more nonspecific unfolding of the protein. When the ORD curve of a protein contains large contributions due to peptide groups in nonhelical conformations or to nonpeptide groups, the change in ORD on exposure will be almost completely because of the gross unfolding of the molecule due to reduced stability after exposure.

Effect of UV Light on Proteins under Denaturing Conditions

As the results described above illustrate, the ORD change on irradiation is quite sensitive to the stability of the irradiated protein at the particular temperature and solvent conditions of the exposure. A model describing how this information could be used in denaturation studies is discussed below in terms of a native protein N which can be denatured by temperature, G·HCl, urea, etc., to some denatured form D:

$$\begin{array}{ccc} N & \overset{K}{\rightleftharpoons} & D \\ h\nu \downarrow & & \downarrow h\nu \\ N^* & \overset{K^*}{\rightleftharpoons} & D^* \end{array}$$

The N^* and D^* are not unique proteins, but mixtures of UV-damaged protein molecules. This discussion will assume that essentially a random mixture of damaged molecules occurs on exposure under each set of conditions and, in the light of the gel electrophoresis data to be presented later in this paper, this seems like a good

assumption. N and D are assumed to be in equilibrium with an equilibrium constant $K = [D]/[N]$. When this equilibrium mixture is irradiated, N^* is formed from N and D^* from D. N^* and D^* are also assumed to be in equilibrium with equilibrium constant $K^* = [D^*]/[N^*]$. The reactions $N \rightarrow N^*$ and $D \rightarrow D^*$ are irreversible.

The fraction of damaged molecules f is given by:

$$f = \frac{[N^*] + [D^*]}{[N] + [D] + [N^*] + [D^*]} = \frac{[N^*](1 + K^*)}{[N](1 + K) + [N^*](1 + K^*)}. \quad (1)$$

Using these equations to solve for $[N^*]$ and $[D^*]$, the following equations are obtained:

$$[N^*] = [N]\left(\frac{1 + K}{1 + K^*}\right)\cdot\frac{f}{1 - f}, \quad (2)$$

$$[D^*] = [N]K^*\left(\frac{1 + K}{1 + K^*}\right)\cdot\frac{f}{1 - f}. \quad (3)$$

At any wavelength the specific optical rotation after exposure $[\alpha]^*$ is given by:

$$[\alpha]^* = \frac{[\alpha]_N[N] + [\alpha]_D[D] + [\alpha]_{N^*}[N^*] + [\alpha]_{D^*}[D^*]}{[N] + [N^*] + [D] + [D^*]}, \quad (4)$$

where $[\alpha]_N$, $[\alpha]_D$, $[\alpha]_{N^*}$, $[\alpha]_{D^*}$ represent the specific optical rotations respectively of N, D, N^*, and D^* at the wavelength being studied. Combination of equations 2–4 gives:

$$[\alpha]^* = \frac{[\alpha]_N + [\alpha]_D K + ([\alpha]_{N^*} + [\alpha]_{D^*} K^*)\left(\frac{1 + K}{1 + K^*}\right)\frac{f}{1 - f}}{(1 + K)\left(\frac{1}{1 + f}\right)}. \quad (5)$$

The specific optical rotation for the unexposed protein solution ($f = 0$), $[\alpha]$, is given by:

$$[\alpha] = \frac{[\alpha]_N[N] + [\alpha]_D[D]}{[N] + [D]} = \frac{[\alpha]_N + [\alpha]_D K}{1 + K}. \quad (6)$$

Solving for $\Delta[\alpha]/[\alpha]$ where $\Delta[\alpha] = [\alpha]^* - [\alpha]$ using these equations gives:

$$\frac{\Delta[\alpha]}{[\alpha]} = \frac{([\alpha]_{N^*} + [\alpha]_{D^*} K^*)\left(\frac{1 + K}{1 + K^*}\right) - [\alpha]_N - [\alpha]_D K}{[\alpha]_N + [\alpha]_D K} \times f. \quad (7)$$

It is obvious that $\Delta[\alpha]/[\alpha]$ is zero when f is zero and is a maximum when f is 1.0.

Below the denaturation region, K and K^* are close to zero and, therefore,

$$\frac{\Delta[\alpha]}{[\alpha]} \cong \frac{[\alpha]_{N^*} - [\alpha]_N}{[\alpha]_N} \times f, \tag{8}$$

which is a pure radiation effect and does not depend on K and K^*. Similarly above the denaturation region K and K^* are both much greater than unity and:

$$\frac{\Delta[\alpha]}{[\alpha]} \cong \frac{[\alpha]_{D^*} - [\alpha]_D}{[\alpha]_D} \times f, \tag{9}$$

which is again a pure radiation effect. If $[\alpha]_D^*$ is approximately equal to $[\alpha]_D$ as with coiled polypeptides, then $\Delta[\alpha]/[\alpha]$ would approach zero above the denaturation region. In the intermediate region a maximum should be obtained in $\Delta[\alpha]/[\alpha]$ if N^* is less stable than N, i.e., K^* greater than K. This is the expected case and is the case obtained with all proteins studied.

In Fig. 3 curves for the hypothetical G·HCl denaturation of N are shown for the case where:

$$N + 10\ \text{G·HCl} \rightleftharpoons D,$$

assuming $[\alpha]_N = -10{,}000$, $[\alpha]_D = -2000$, $[\alpha]_{N^*} = -7500$, $[\alpha]_{D^*} = -2000$, $K = 9.8 \times 10^{-4}[\text{G·HCl}]^{10}$, $K^* = 5K$, and $f = 0.25$.

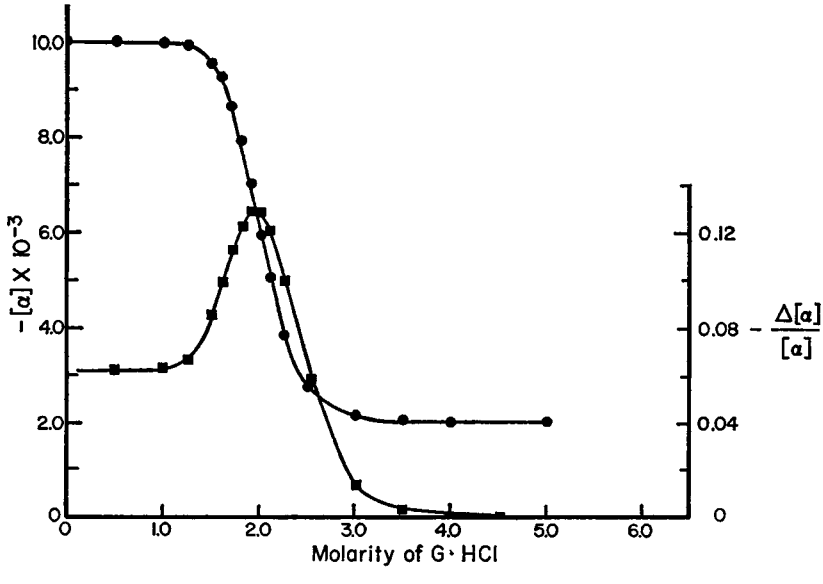

FIGURE 3 The effect of G–HCl on the sensitivity of a model protein N to exposure by 225 nm radiation. $[\alpha]$, ●, and $\Delta[\alpha]/[\alpha]$, ■, as a function of G·HCl molarity. See the text for details of the model.

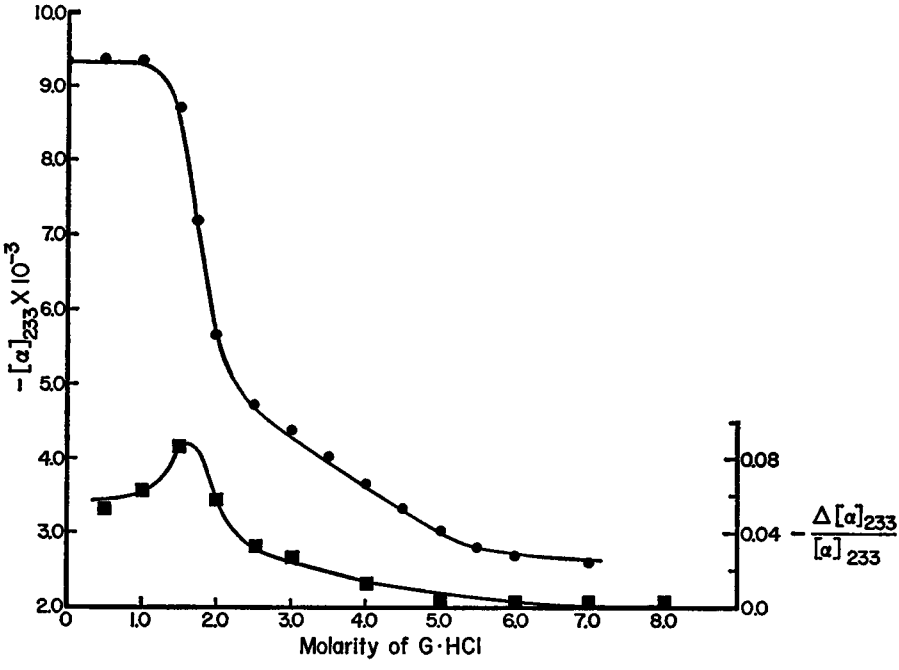

FIGURE 4 Effect of G–HCl on the sensitivity of BPA to exposure by 225 nm radiation in the Cary 60 spectropolarimeter. $[\alpha]_{233}$, ●; $\Delta[\alpha]_{233}/[\alpha]_{233}$, ■. The points were determined experimentally while the lines drawn for both curves are those calculated from the model presented in the text. No time dependence was found for the rotation of the unexposed sample.

The general shape of these curves stays essentially the same over a wide range of conditions, with only the position of the maximum and the relative heights being shifted to correspond to the particular conditions of the denaturation being studied.

The G·HCl denaturation of BPA cannot be satisfactorily fitted by this simple two-state model; however, if the denaturation is assumed to consist of two essentially noninteracting steps, excellent fit can be obtained. This is shown in Fig. 4. The lines in Fig. 4 were calculated using a computer program to give the best fit to the experimental data assuming the following model:

$$\begin{array}{ccccc} N + 10\ \text{G·HCl} & \overset{K_1}{\rightleftharpoons} & D_1 + 5\ \text{G·HCl} & \overset{K_2}{\rightleftharpoons} & D_2 \\ \downarrow h\nu & & \downarrow h\nu & & \downarrow h\nu \\ N^* + 10\ \text{G·HCl} & \underset{K_1^*}{\rightleftharpoons} & D_1^* + 5\ \text{G·HCl} & \underset{K_2^*}{\rightleftharpoons} & D_2^* \end{array}$$

Numerical values chosen for the various parameters were: $K_1 = 3.72 \times 10^{-3}[\text{G·HCl}]^{10}$, $K_1^* = 5K_1$, $K_2^* = K_2 = 9.76 \cdot 10^{-4}[\text{G·HCl}]^5$, $f = 0.25$, $[\alpha]_N = -9300$, $[\alpha]_{N^*} = -7100$, $[\alpha]_{D_1} = -4700$, $[\alpha]_{D_1^*} = -4200$, $[\alpha]_{D_2} = [\alpha]_{D_2^*} = -2500$.

The results clearly show the distinct two-step nature of the transition, the structure of the intermediate D_1 being quite stable. This stability is indicated by the high concentration of G·HCl required for the second step and is also reflected in the relative sizes of K and K^*. In the first transition K_1^* is much greater than K_1 indicating an extreme lability toward photolysis. In the second transition, however, K_2 and K_2^* are approximately equal indicating that D_1 is not only more resistant to G·HCl denaturation, but is also quite resistant to conformational changes resulting from photolysis. The zero value of $\Delta[\alpha]/[\alpha]$ in 6.0 M G·HCl would also indicate that the BPA conformation is that of a random coil in this solvent, in agreement with Tanford (1968).

Thermal Denaturation of BPA and the Effect of 225 nm Irradiation

The thermal denaturation of BPA has also been studied using UV exposure at 225 nm. In Fig. 5 $\Delta[\alpha]_{233}/[\alpha]_{233}$ values are plotted at various temperatures along with $[\alpha]_{233}$, which is a standard thermal denaturation curve. This study was complicated by the fact that at temperatures above about 70°C the rotation of the protein is time dependent even in the absence of radiation. This is presumably caused by aggregation of the denatured molecules. The curve for $\Delta[\alpha]_{233}/[\alpha]_{233}$ must be corrected for these changes and this is done in Fig. 5 where the dashed line represents

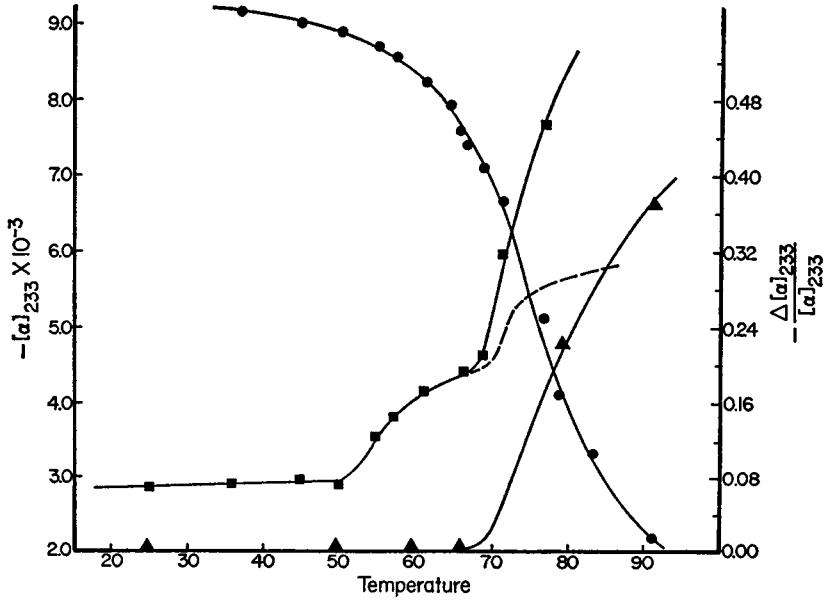

FIGURE 5 Effect of temperature on the sensitivity of BPA to exposure by 225 nm radiation in the Cary 60 spectropolarimeter. $[\alpha]_{233}$, ●; $\Delta[\alpha]_{233}/[\alpha]_{233}$, ■, and the change in $\Delta[\alpha]_{233}/[\alpha]_{233}$ on storage above 70°C in the absence of radiation, ▲. The dashed line represents changes due to exposure only as described in the text.

the change caused only by irradiation. This procedure could lead to considerable errors in the exact magnitude of the resultant curve, but the gross shape of the curve should be correct.

From these curves it would seen that there is a reversible unfolding of the BPA molecule between 51 and 68°C. A second more drastic unfolding of the protein begins at 68°C. It should be noted that the curve of $[\alpha]_{233}$ vs. temperature is smooth and would indicate only one broad transition. The curves of $\Delta[\alpha]_{233}/[\alpha]_{233}$, however, are more informative about the number of steps in the transition and clearly inticate that there are at least two with midpoints around 60 and 75°C. In this case the second transition could also have been detected by the irreversible changes in the protein.

Gel Electrophoresis Experiments on Exposed BPA

The intrinsic viscosities of polyamino acids after exposure indicated rather drastic molecular weight decreases and it would be of interest to ascertain whether peptide cleavage also occurs in proteins on exposure. Also it would be helpful to determine a quantum yield for peptide bond destruction so that the amount of photolysis could be predicted for a particular experiment. A convenient method for answering these questions involves denaturing the exposed proteins with SDS, reducing the disulfide bonds with β-ME, and analyzing the resulting peptides on polyacrylamide gels (Weber and Osborn, 1969). If a bond in the peptide backbone is cleaved in a protein on exposure, lower molecular weight peptides will be formed which will migrate faster than the intact protein on the gels. The amount of peptide destruction can also be determined by following the amount of intact protein remaining on the gels after exposure.

Samples of a stock BPA solution (0.1%, pH 5.5, 0.1 M NaCl) were exposed in the Bausch & Lomb instrument for 0, 2, 3, 5, 10, and 25 hr, and analyzed on SDS gels. In Fig. 6 tracings of some of the patterns obtained by scanning the gels are shown. All gels had 50 μl of solution applied which before exposure would have contained 25 μg of BPA. Peptides of molecular weight lower than BPA have a higher mobility and appear towards the bottom of the gel (which is to the right of the scans shown in Fig. 6). It is evident from this figure that UV light is breaking bonds in the polypeptide backbone of BPA and giving lower molecular weight peptides.

In later experiments to quantitate the amount of native protein remaining after exposure the amounts of samples applied to the gels were varied, depending on exposure time, so that the amount of native BPA on each gel would be fairly constant. It was found that a curve of area under a gel peak vs. micrograms of protein applied to the gel varied somewhat from one run to the next, presumably because of slight differences in staining and destaining. For this reason, with each set of gels a standard graph of area vs. micrograms had to be prepared using unexposed BPA.

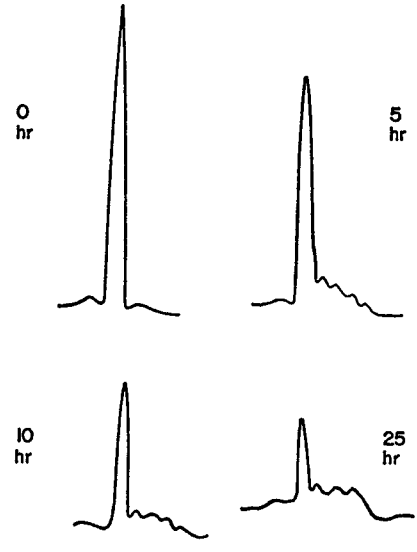

FIGURE 6 The effect of 225 nm irradiation on the SDS-β-ME gel pattern of BPA. Tracings of gel scans from the Gilford spectrophotometer with hours of exposure indicated for each sample. The samples exposed 0–10 hr were scanned with 1.0 absorbance units full scale, while the 25 hr sample was scanned with 0.5 absorbance units full scale.

The exposed samples were also scanned and the area under the remaining native BPA peak was determined; the micrograms of native BPA could then be found from the standard graph.

In Fig. 7 the natural logarithm of the initial concentration of native protein, c_0, divided by the concentration c, after some time of exposure ($\ln c_0/c$) is plotted as a function of exposure time. The disappearance of native protein is first-order within experimental error as it should be if the absorbance of the products is approximately the same as for native BPA. From the slope of the line the first-order rate constant can be found and the quantum yield for peptide bond photolysis calculated in the following manner:

$$\phi = \frac{-dc/dt}{I_0\, c/c_0 \cdot f_a}, \tag{10}$$

where ϕ = quantum yield, t = time of exposure, I_0 = incident light intensity, c_0 and c are the protein concentrations at time zero and time t respectively, and f_a = the fraction of light absorbed. For a first-order reaction, $dc/dt = -k \cdot c$, and hence

$$\Phi = kc_0/I_0 f_a\,. \tag{11}$$

I_0 for these conditions (225 nm) is $4.66 \cdot 10^{-11}$ einsteins/sec ml; k is found from the slope in Fig. 7, $2.64 \cdot 10^{-5}$/sec; and c_0 is $7.59 \cdot 10^{-9}$ moles/ml; f_a is essentially 1.0 for

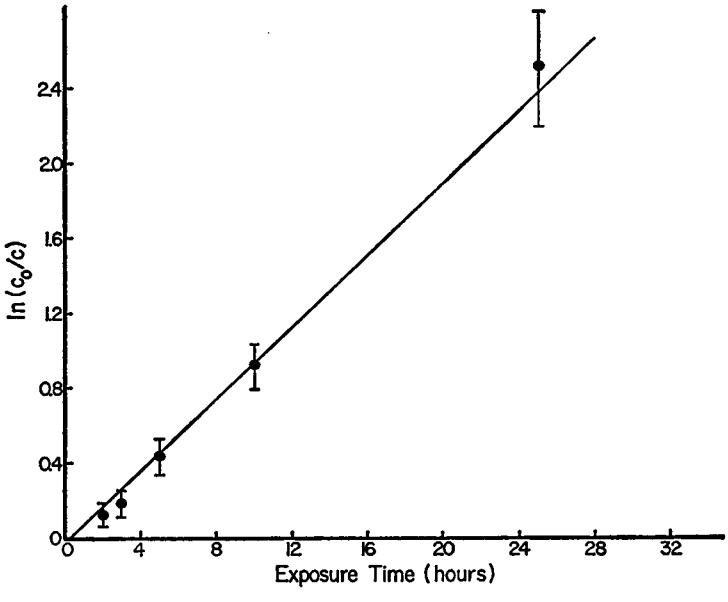

FIGURE 7 First-order rate plot for the photolysis of native BPA on exposure to 225 nm radiation.

this concentration and wavelength, and this gives, on substitution into equation 11, $\phi = 4.3 \cdot 10^{-3}$ moles/einstein or molecules/quanta.

The significance of energy transfer in protein photochemistry is an important and as yet unresolved question (Smith and Hannawalt, 1969). The results shown in Fig. 6 would suggest that a rather broad range of products is obtained on exposure of BPA, indicating rather low specific cleavage. There are some very light bands visible on the gels and these show up in Fig. 6 as bumps on the background of nonspecific products. These bumps or bands never reach a very high concentration because of their slow rate of formation and fairly rapid rate of breakdown. These small bands indicate that some energy transfer from aromatic groups to peptide bonds probably occurs, but it seems fairly small in magnitude when compared to nonspecific peptide bond photolysis.

Since at 225 nm the peptide absorption accounts for approximately half of the light absorbed by BPA, the quantum yields for peptide absorption only, ϕ_{pep}, is approximately 9×10^{-3} assuming that energy transfer does not account for a significant amount of cleavage in the peptide chain. ϕ should vary from protein to protein depending on the amount of aromatic residues a particular protein contains, but ϕ_{pep} should be fairly constant from one protein to the next if there is no great difference in energy transfer from aromatic groups to the peptide groups among the proteins studied.

It should be mentioned that BPA has a rather low ratio of tyrosine and tryptophan residues to peptide bonds and it was felt that proteins with a higher ratio

might show more evidence of energy transfer. Two other proteins, chymotrypsinogen and β-lactoglobulin, were exposed and analyzed by the gel method, but no evidence of specific peptides produced in appreciable concentrations could be found after exposure of these proteins.

Destruction of Proteins in Spectropolarimetry

The equation discussed above can be used to predict the amount of protein destruction obtained in instruments of the Cary 60 type when scanning protein solutions. Rearranging equation 10 and integrating gives

$$\ln (c_0/c) = \frac{(\Phi I_0 f_a)t}{c_0}. \qquad (12)$$

For exposure in the 2.0 cm jacketed cell at 233 nm in the Cary 60 instrument, I_0 was 2.83×10^{-12} einsteins/ml sec for the conditions used, f_a was 0.82, the BPA concentration used was 7.59×10^{-10} moles/ml, and the quantum yield was 4.6×10^{-3} as discussed above. Substituting into equation 12 gives:

$$\ln (c_0/c) = (1.41 \times 10^{-5}) \times t, \qquad (13)$$

where t is in seconds. For a 1 hr exposure in the Cary 60 spectropolarimeter, $\ln c_0/c = 0.051$, corresponding to a 5% destruction of the native protein. This is also the change in rotation that would be obtained if the specific rotation of the reacted protein is zero at 233 nm. This situation is approached for BPA in G·HCl or at higher temperatures, but when the protein does not completely unfold on exposure less than 5% change in specific rotation would be found in 1 hr. This change is for the entire cell, however, and is the maximum change that would be found if a protein sample were exposed and stirred before recording $[\alpha]_{233}$. If $[\alpha]_{233}$ is monitored by constant exposure and recording then larger changes would be found since only the more highly reacted protein in the light beam would be monitored.

Protein concentration is also an important variable and its effect can be demonstrated by the following calculation. If the protein concentration is increased by a factor of 5 (an easily obtainable concentration), the fraction of light absorbed increases only from 0.82 to about 1.0. This gives $\ln c_0/c = 0.012$, or only slightly more than a 1% change as compared with a 5% change at the lower concentration.

The diameter of the cells used in the Cary 60 spectropolarimeter also has quite a striking effect on the amount of ORD change obtained on exposure, as shown in Fig. 8. The difference between the cells is even greater when the samples are exposed and stirred before recording ORD since the 22 mm cell has a much larger volume not exposed to the light beam. The complicated nature of the curve is a consequence of the effects of protein diffusion into and out of the light beam being superimposed on the protein photolysis. The expected difference between the cells can be calcu-

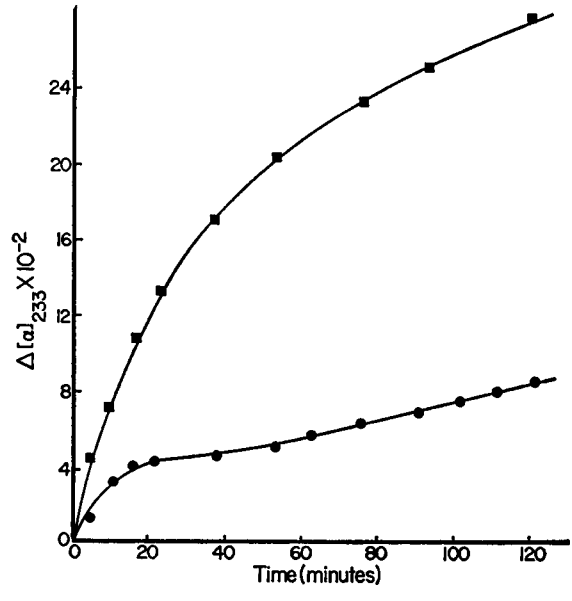

FIGURE 8 Effect of cell size on the change in $[\alpha]_{233}$ on exposure of BPA in 1.5 M G·HCl to 233 nm radiation in the Cary 60 spectropolarimeter. 8.0 mm diameter jacketed cell, ■; 22.0 mm diameter cell, ●. The slit was opened to 2.4 mm to obtain easily measurable changes in short time periods. The normal slit setting at 233 nm was 1.5 mm (1.5 nm spectral bandwidth).

lated using their respective volumes. The jacketed cell had a diameter of 8.0 mm while the other cell had a diameter of 22.0. Since both cells were 2.0 cm in length, the ratio of volumes and total protein content is 0.132. The cells absorb the same number of quanta in a given exposure time, and in a solution stirred after exposure, the jacketed cell would, therefore, show approximately 7.5 times more change in ORD than the large cell. The difference between the cells on constant exposure and recording as shown in Fig. 8 is not this large since mixing is inefficient, but there is still a large difference between the two cells.

A larger cell is obviously preferable for minimizing the light reaction, but the smaller cells are better for thermal studies where considerable time must be spent waiting for thermal equilibrium. Smaller cells are also desirable in cases where protein samples may be in short supply, and are almost mandatory for kinetic studies in thermal denaturation experiments where the temperature in the cell must be changed quite rapidly. The choice of cells must then be a compromise.

Exposing native BPA at 25°C (rather than BPA in 1.5 M G·HCl) gives much smaller changes which are difficult to detect even in the jacketed cell (Fig. 1 of Wilson and Foster, 1970). Exposing native BPA at 25°C in the 22.0 mm diameter cell gives changes which are hardly noticeable, but as pointed out above, this does not mean that the protein is not reacting, merely that it is unfolding to only a small

extent under these conditions. If an exposed protein solution is heated as in a thermal denaturation experiment, the reacted protein will begin unfolding sooner than the unreacted protein giving an artificially broadened transition curve. These errors would be largely masked in many cases until the protein reached the threshold of denaturation and could cause incorrect interpretations of the data when analyzing for the two-state nature of a transition or in calculating the various thermodynamic parameters from the transition curve.

From the data given above some conclusions on the use of high intensity light source spectropolarimeters are possible. Workers scanning protein solutions with normal speeds can use small diameter cells with safety. When doing very slow scans or a number of scans on the same solution, large diameter cells should be used where possible. Workers studying thermal denaturation by constant recording of optical rotation in the peptide absorption region should be particularly aware of the risk of obtaining artificially broadended curves.

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REFERENCES

BEYCHOK, S. 1968. *Annu. Rev. Biochem.* **37**:437.

DAVIDSON, B., and G. D. FASMAN. 1967. *Biochemistry.* **6**:1616.

MCLAREN, A. D., and D. SHUGAR. 1964. Photochemistry of Proteins and Nucleic Acids. Pergamon Press Ltd., Oxford, England.

MEYBECK, A., and J. MEYBECK. 1967. *Photochem. Photobiol.* **6**:355.

MEYBECK, A., and J. J. WINDLE. 1969. *Photochem. Photobiol.* **10**:1.

PACE, N. C., and C. TANFORD. 1968. *Biochemistry.* **7**:198.

SHUGAR, D., A. ADAMIEC, and E. SZTUMPF. 1959. *Acta Biochim. Pol.* **6**:417.

SMITH, K. C., and P. C. HANNAWALT. 1969. Molecular Photobiology. Academic Press, Inc., New York.

TANFORD, C. 1968. *Advan. Protein Chem.* **23**:121.

TIMASHEFF, S. N., R. TOWNEND, and L. MESCANTI. 1966. *J. Biol. Chem.* **241**:1863.

TOWNEND, R., T. F. KUMOSINSKI, and S. N. TIMASHEFF. 1967. *J. Biol. Chem.* **242**:4538.

WEBER, K., and M. OSBORN. 1969. *J. Biol. Chem.* **244**:4406.

WILSON, W. D., and J. F. FOSTER. 1970. *Biochem. Biophys. Res. Commun.* **38**:552.

WILSON, W. D., and J. F. FOSTER. 1971. *Biochemistry.* **10**:1772.