PHOTOCHEMISTRY OF PROTEINS

XV. SOME OBSERVATIONS ON THE ULTRAVIOLET ABSORPTION SPECTRA OF PROTEINS*

by

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ANSLOW AND NASSAR[1], in studying the absorption spectrum of gelatin, believed that they had found evidence for an absorption band near 2800 A by the peptide bond. We have shown elsewhere that the band observed near 2800 A in gelatin cannot be due to the peptide bond, since no such band is found in clupein hydrochloride[2]. ANSLOW now admits that this band cannot be due to the peptide bond, since it does not appear in linear amides[3]. This small, but definite, absorption band near 2800 A, which is shifted toward the red in alkaline solution[1], is shown here to be due to quite small amounts of tyrosine, known from recent studies to be found in gelatin samples of several origins.

RIDEAL AND ROBERTS[4] believe that they have evidence showing that approximately 5% of the radiation absorbed by protein at wave-length 2537 A is directly absorbed at the keto-imino linkage. Our results indicate that this estimate is probably too high and that the value is probably nearer 2–3% in most proteins. This conclusion is based on the ultraviolet light absorption of the hemipeptide acetylalanine and a comparison of the absorption spectra of some proteins (chymotrypsin, insulin, gelatin and examples from the literature) and their corresponding mixtures of amino acids. A comparison of the effect of irradiation on a polypeptide and a mixture of peptides of similar absorption spectra properties is also presented.

EXPERIMENTAL AND RESULTS

For methods of measurement of spectra, reference may be made to previous papers in this series.

Gelatin. Gelatin ("extra pure", Aktieselskabet Dansk Gelatin Fabrik, Copenhagen) was dialyzed against distilled water and dissolved in water with a resultant pH of 4.70. The final concentration was 0.965 mg nitrogen per ml. The absorption curve, Fig. 1, shows slight maxima at 2580, 2640 and 2680 A, which are definitely due to phenylalanine. The maxima at 2750 and 2815–2840 A are definitely those of combined tyrosine[5].

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Gelatin from several sources is known to contain *ca* 2% phenylalanine and 0.2–0.9% tyrosine[6]. At the same concentration, at pH 12.6, a shift of the spectrum to longer wave lengths is observed. The results with gelatin are not corrected for light scattering[4].

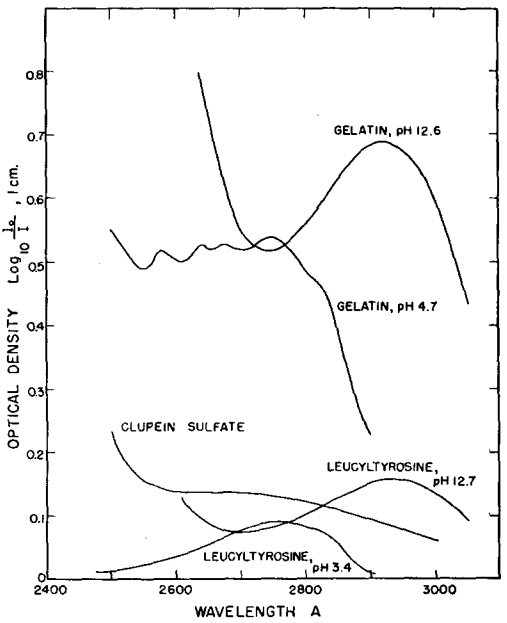

Fig. 1. Ultraviolet absorption spectra of gelatin, clupein and leucyltyrosine.

The optical densities of $5 \cdot 10^{-5}$ *M* solutions of L-leucyl-L-tyrosine[7] are also shown in Fig. 1 at pH 3.4 and pH 12.7. It will be observed that an identical shift in maxima occurs with a change from acid to alkaline reaction. Thus there is no need to postulate a special absorption to any grouping in the 2750–2800 A region, such as to the peptide bond. The absorption data of leucyltyrosine may be used to calculate a value of 0.71% tyrosine in our gelatin sample, *cf.* ref. 6.

Further evidence that the peptide bond has no special absorption in the 2800 A region is furnished by an examination of clupein sulfate, Fig. 1, 10 mg per ml in water. (The clupein sulfate was kindly furnished by Dr C. F. JACOBSEN.)

Chymotrypsin. A comparison of the absorption of chymotrypsin (pH 5.0) with an amino acid mixture of the same residual make-up as the zymogen[8] is of interest in revealing that the absorption by the peptide bond at 2537 A in a protein is small. In the previous paper[8] no correction was made, in plotting the data for the amino-acid mixture, for the water of condensation, which is theoretically eliminated in formation of the protein. The apparent optical densities of the mixture per mg per ml must be multiplied by 112.36/94.98, since 94.98 g of amino-acid residues experimentally give rise to 112.36 g of amino acids[9]. In other words what is needed is the optical density of a mg of protein per ml and the density of a mg of amino-acid residues (uncombined) per ml. In Fig. 2 we have prepared these plots for both solutions. It will be seen that at 2537 A and at 2500 A the absorption by the mixture is actually greater than by the protein. This is in accordance with the findings of HAUROWITZ AND ASTRUP[10] with ox serum globulin and the corresponding enzymic hydrolysate and with those of BEAVEN *et al.*[11], with horse serum globulin and the corresponding peptic digest. A correction for light scattering is not necessary for chymotrypsin[12].

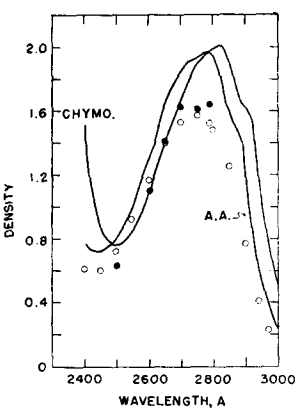

Fig. 2. Ultraviolet absorption spectra of chymotrypsin, the corresponding mixture of amino acids (A. A.), calculated values for the more strongly absorbing amino acids (solid circles) and found values for a mixture of the more strongly absorbing amino acids (open circles).

Insulin. The spectrum of insulin has been determined by several workers[4,13,14]. For a molecular weight of 36,000, the molecular extinction coefficients found by various

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TABLE I

MOLECULAR EXTINCTION COEFFICIENT DATA FOR INSULIN

Insulin	*Maximum*	*Minimum*	*References*
	35,200	15,650	13
	34,600	16,400	14*
	34,840	16,900	**
Calculated for Insulin	34,980	11,700	14*

* The values in ref. 14 (Table II) are incorrectly given due to arithmetic errors (E. H. KAPLAN, private communication).

** Same sample as in ref. 14, amorphous insulin, unpublished data of S. MALAMENT.

authors are tabulated in Table I. Values calculated in the usual way from the amino-acid composition are also shown, using the amino-acid data of BRAND[15] and the extinction coefficients of FROMAGEOT AND SCHNEK[16]. As noted by CRAMMER AND NEUBERGER, the agreement between calculated and found values at the maximum is satisfactory. The agreement at the minimum is poor, however. RIDEAL AND ROBERTS ascribe this difference to absorption by the peptide bond in the neighborhood of 2500 A. In view of our results with chymotrypsin and the corresponding amino-acid mixture*, which agreed more closely at the minimum, we have compared the absorption of insulin with that of the corresponding amino-acid (A. A.) mixture, Fig. 3. The insulin solution contained 1 mg per ml of insulin (dried over P_2O_5) in 0.0031 6 *N* HCl (pH 2.58). The amino acid solution contained 1 mg per ml of amino acids in the same solvent (pH 2.67). The optical densities** for a 1 cm path length were multiplied by 1.167. The factor 1.167 is the ratio of the weight of amino acids obtained from 100 g insulin to the weight of amino-acid residues accounted for in insulin[15]. It will be seen that the two curves more nearly coincide in the region of 2600 to 2725 A than do those of RIDEAL AND ROBERTS. Their calculated curve is almost identical with the curve for the amino-acid mixture; their maximum is the same height as ours. The agreement at 2500 A is also good. Their maximum density for insulin gives a molecular extinction coefficient of *ca* 37,800, which is higher than found by others, however (*cf.* Table I). The difference in density at the protein minimum and the amino-acid curve is about the same in their study as in ours.

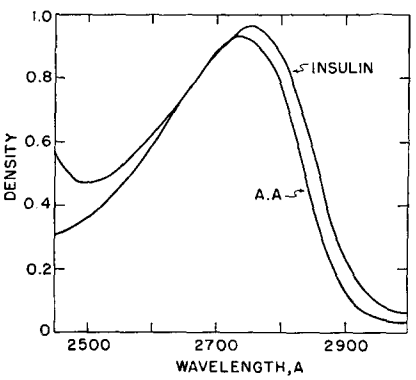

Fig. 3. Ultraviolet absorption spectra of insulin and of the corresponding amino acid mixture.

Pancreatic trypsin inhibitor (PTI). In Fig. 4a, the optical density per cm path length is plotted for a solution of pancreatic trypsin inhibitor***, 1 mg per ml (not corrected

* Amino acids from Mann Fine Chemicals, Inc., N.Y. 6, N.Y., and Eastman Kodak Company, Rochester, N.Y.

** Obtained with micro cells, 75 cmm.

*** Worthington Biochemical Laboratory, Freehold, N.H., non-crystalline lyophilized, with 75.2% ammonium sulfate and 4% water (*cf.* M. KUNITZ AND J. H. NORTHROP, *J. Gen. Physiol.*, 19 (1936) 991).

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for moisture or ammonium sulfate) in $M/15$ phosphate buffer, pH 3.05. Also shown is the density of a solution irradiated for 5½ minutes (2.8 ml in air, at an intensity of *ca* $0.15 \cdot 10^{-4}$ einsteins per ml per hour at 2537 A) after standing for 24 hours at 5° following irradiation. Using 1,300 as the extinction coefficient of tyrosine[17] at 2750 A and our density of 0.216, one can calculate a concentration of tyrosine residues of $0.166 \cdot 10^{-6}$ M per ml. Taking the molecular weight[9] of the inhibitor as 6,000 the inhibitor concentration was $0.208 \cdot 10^{-3}/6000$ or $0.347 \cdot 10^{-7} M$/ml. Thus, the calculated amount of tyrosine residues in the molecule is 0.166/0.0347 = 4.8 or roughly 5.

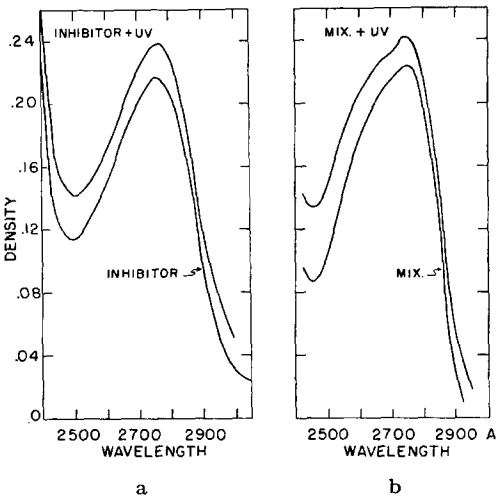

Fig. 4. Ultraviolet absorption spectra of (a) irradiated and unirradiated trypsin inhibitor and (b) irradiated and unirradiated solutions of a mixture (mix) of leucyltyrosine and propionylphenylalanine.

For comparison, a solution of leucyltyrosine was made up to give a density at 2760 A equaling that of the PTI solution; propionylphenylalanine[7] was added to nearly duplicate the absorption of the PTI, Fig. 4b, throughout the spectrum. The final solution was $0.548 \cdot 10^{-6}$ M per ml in phenylalanine residues. This solution was also subjected to 5½ minutes of irradiation at 2537 A. Irradiation produced an increase in absorption of both the PTI and the corresponding mixture. The increase for the mixture is about one to one and one-half times that for the inhibitor (compare Fig. 4a and Fig. 4b). The indication, however, is that the behavior of the mixture is similar to that of the inhibitor with regard to the photochemical reactions[18].

As was to be expected, the maximum absorption of the PTI was shifted to longer wave lengths at pH 12.7 (density of 0.242 at 2880–2890 A at 1 mg per ml uncorrected for ammonium sulfate and water in the Worthington preparation).

A preliminary value for the quantum yield for inactivation of PTI at 2537 A, based on loss of ability to inhibit the proteolytic activity of trypsin, is 0.031 at pH 4.5 in $M/15$ phosphate buffer. The value is subject to considerable experimental uncertainty.

DISCUSSION

By reference to Fig. 2, it will be seen that at 2537 A and at 2500 A the absorption by the mixture of amino acids is actually greater than by the protein in the case of chymotrypsin. Obviously, a calculation of the fraction of absorbed light at these wavelengths absorbed by the CONH linkage by comparison of these curves cannot be performed. RIDEAL AND ROBERTS have attempted such comparisons with serum albumin, gelatin and insulin, by a comparison of calculated absorption of the more strongly absorbing chromophores (amino acids) with the corresponding protein spectra. They found that the calculated curves were below the curves for the proteins at 2500 and 2800 A but more nearly coincided with the protein curves between 2600 and 2700 A.

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It seemed to us that part of the discrepancy with their procedure might arise from a lack of consideration of the *ca* 80% non-aromatic residues (by weight) in proteins. We therefore have obtained for comparison the absorption spectrum of a mixture of phenylalanine, tyrosine, tryptophan, cysteine and cystine in the proportions[8,9], in which they are found in the protein and to the extent they occur in 1 mg per ml of the total amino-acid mixture (corrected for water of condensation), Fig. 2. Also, for comparison the densities to be expected for these five amino acids have been computed from the molecular extinction data of FROMAGEOT AND SCHNEK (also corrected for water of condensation). These last two curves agree fairly well, but they fall considerably below the curve for the total amino-acid mixture. A correction for water of condensation brings the density to only 1.6 at 2750 A for both. The 83% by weight of the other amino acid residues in the BRAND mixture must therefore contribute appreciably to the absorption of the protein and to the complete amino-acid mixture.

RIDEAL AND ROBERTS claim that a large discrepancy exists between calculated and found values for the absorption spectrum of gelatin (found values corrected for light scattering). They give an $E^{1\%}_{1\,cm}$ of *ca* 0.64 at 2500 A, 0.62 at 2600 A and 0.55 at 2700 A for gelatin but values of somewhat less than 2/3 of these for their calculations. Unfortunately, they did not give the amino-acid composition assumed for their calculations and apparently they ignored, as did ANSLOW, the spectroscopically significant amounts of tyrosine known to be present in gelatin[6]. We have determined the spectrum of an amino-acid mixture corresponding to gelatin[6] in water at pH 4.55, which gave 0.61 at 2500 A, 0.70 at 2600 A and 0.80 at 2700 A for a 1% solution (the amino-acid mixture was as given by NEUMAN[6] and contained hydroxyproline, 13% (R. J. BLOCK AND D. BOLLING, "*Amino-Acid Composition of Protein and Foods*", C. C. Thomas, Springfield, Illinois, 1945); tyrosine, 0.41%; and phenylalanine, 2.3% of total amino-acid mixture, as well as the other amino acids listed by NEUMAN). The amount of tyrosine chosen was arbitrarily about the average of that reported for several gelatin samples by NEUMAN. Thus, in the region of 2700 A RIDEAL's results and ours could only agree by accident. Between 2500 and 2600 A (absorption by phenylalanine residues) the agreement between our amino-acid mixture and the experimental curve for gelatin of RIDEAL AND ROBERTS is good, and there is no need for assigning 5% or more of the total absorption to absorption by CONH at 2537 A.

Incidentally, a solution of the amino acids at the same concentration and at pH 12.5 showed the same kind of shift of maximum toward the red as was shown by intact gelatin and to the same extent (maximum at 2920 A).

An examination of the protein and amino-acid absorption curves of RIDEAL AND ROBERTS for bovine serum albumin shows virtually no difference in optical density values at 2537 A. This datum, coupled with similar work reported here with chymotrypsin and elsewhere on ox and horse globulin, referred to above, leads one to conclude that there is little evidence from which it can be concluded that approximately 5% of the total absorption at 2537 A is due to CONH linkages in intact proteins.

One can deduce, however, in another way, that the CONH linkage in a protein absorbs *ca* 2–3% of the light absorbed near 2537 A. In a typical protein absorption by a structure like clupein (aliphatic polypeptide) would be one to 3% of that of a protein between 2525 and 2800 A. For example, the density of clupein sulfate in this region, 10 mg per ml, is roughly 0.15; that of a typical protein at the same concentration is 5–8, or greater.

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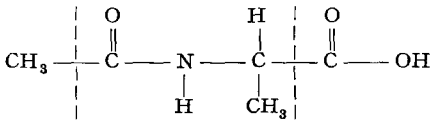
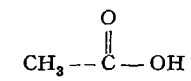
We can calculate the per cent. of the absorbed light absorbed by –CONH– in chymotrypsin in the following way:

For acetylalanine[19]:



the molecular extinction coefficient, ε, is 1.28 at 2537 A.

For acetic acid[19]:



ε_{2537} = 0.02 (the combination of other groups, such as NH_2, to the carboxyl to form an amide fails to alter appreciably the absorption spectra of the compound[20]). Since the carboxyl is insulated[20] from the peptide group in acetylalanine by the $H-\overset{|}{C}-CH_3$ group, we may subtract 0.02 from 1.28 and obtain ε_{2537} = 1.26 for the peptide bond at 2537 A. The $CH_3-\overset{|}{C}H-$group is transparent at this wave length[20]. To determine the per cent. absorption by the peptide linkage we may consider that for a molecular weight of 36,800 there are *ca* 360 such linkages in chymotrypsin[21]. Thus, ε_{2537} (for CONH) = 360 × 1.26 = 454. For this molecular weight, ε_{2537} = 30,500. The % absorption by the peptide bond is thus only 454 × 100/30,500 = 1.5%.

The absorption of light of 2537 A at CONH linkages is probably only about 1 to 3% in most proteins, an estimate of about half that suggested by RIDEAL AND ROBERTS.

The well-known[5,22] absorption of ultraviolet light by the peptide bond at wave lengths shorter than 2300 A has been recently confirmed[23]. Increased absorption by the CONH linkage at these shorter wave-lengths, followed by photolysis, could account in part for the increase in effective quantum yields for inactivation of enzymes in this region of the spectrum[5]. It must be remembered, however, that internal photosensitization by aromatic groups[19] can also lead to photolysis of the peptide bond. It has been assumed that such photolysis leads to inactivation—denaturation of proteins[5,19]. Evidence is also accumulating that modification of an aromatic ring can lead to inactivation, which may be the more important mechanism near and at the 2537 A Hg line (paper in preparation). It also seems likely that some particular group or aromatic residue may be more important photochemically than others in leading to loss of biological activity in enzymes and other protein molecules[24].

SUMMARY

The ultraviolet absorption spectra of chymotrypsin, insulin, pancreatic trypsin inhibitor, gelatin and clupein sulfate are presented. Comparisons are made between absorption spectra of corresponding mixtures of amino acids and chymotrypsin, insulin, gelatin and pancreatic inhibitor. From such comparisons, and calculations based on data in the literature, it is concluded that of the light absorbed at 2537 A by typical proteins, about 1–3% of the total absorption is by the peptide linkage. The percentage is much higher at *ca* 2000–2300 A.

The effect of ultraviolet light (2537 A) on the absorption spectrum of trypsin inhibitor and of a corresponding mixture of peptides is similar. A preliminary value of the quantum yield for inactivation of the inhibitor is 0.031.

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RÉSUMÉ

Les auteurs présentent les spectres d'absorption dans l'ultraviolet des substances suivantes: chymotrypsine, insuline, inhibiteur pancréatique de la trypsine, gélatine et sulphate de clupéine. Ils établissent des comparaisons entre des mélanges correspondants d'acides aminés et la chymotrypsine, l'insuline, la gélatine et l'inhibiteur pancréatique. Par de telles comparaisons et des calculs basés sur des données de la littérature ils arrivent à la conclusion que environ le 1–3 % de la lumière totale absorbée à 2537 A par des protéines typiques est absorbé par la liaison peptidique. Ce pourcentage est beaucoup plus élevé à 2000–2300 A environ.

L'effet de la lumière ultraviolette (2537 A) sur le spectre d'absorption de l'inhibiteur de la trypsine et d'un mélange correspondant d'acides aminés est semblable. Une valeur prélimininaire du rendement quantique pour l'inactivation de l'inhibiteur est 0.031.

ZUSAMMENFASSUNG

Die Ultraviolett absorptions-Spektren von Chymotrypsin, Insulin, Pancreas-Trypsinhemmstoff, Gelatin und Clupeinsulphat werden gezeigt. Vergleiche zwischen den Absorptionsspektren von entsprechenden Mischungen von Aminosäuren und Chymotrypsin, Insulin, Gelatin und Pankreashemmstoff werden angestellt. Auf Grund solcher Vergleiche und von Berechnungen mit Angaben aus der Literatur wird geschlossen, dass ungefähr 1–3 % der Gesammtmenge des von typischen Proteinen bei 2537 A absorbierten Lichtes durch die Peptidbindung absorbiert wird. Dieser Prozentsatz ist bei ca 2000–2300 A viel höher.

Die Wirkung des ultravioletten Lichtes (2537 A) auf das Absorptionsspektrum von Trypsinhemmstoff und von einer entsprechenden Aminosäuremischung ist ähnlich. Ein vorläufiger Wert für die Quantenausbeute der Inaktivierung des Hemmstoffes ist 0.031.

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