PHOTOCHEMISTRY OF PROTEINS

VIII. INACTIVATION OF INSULIN BY ULTRAVIOLET LIGHT*

by

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In continuation of the study of the effect of ultraviolet light on various proteins the irradiation of insulin has been investigated.

Shortly after its isolation NITZESCU[1] found that insulin was not inactivated by ultraviolet irradiation. This was probably because the radiation was absorbed by the glass container, as ELLIS AND NEWTON[2] observed that insulin was not inactivated when irradiated in a glass tube, but was inactivated when irradiated in a quartz tube. The absorption spectrum of insulin and the effect of ultraviolet light on the hormone have since been the subject of many investigations[3–15]. A quantitative study of the effect of ultraviolet irradiation has not yet been carried out, and since insulin of high purity was available, it seemed desirable to determine the quantum yield for the inactivation of the hormone, and if possible to correlate the loss in activity with chemical changes in the molecule.

The absorption spectrum of insulin has been shown[9, 15] to be the resultant of the absorption of the tyrosine and the cystine in the molecule. KUHN, EYER, AND FREUDENBERG[9] observed that the change in the absorption spectrum upon irradiation of insulin was similar to the change upon irradiation of a mixture of tyrosine and cystine in the proportions in which they are present in insulin. When a solution of tyrosine was irradiated, the absorption increased at the minimum (2500 Å) and was unchanged at the maximum (2750 Å) of its absorption spectrum. Irradiation of a cystine solution resulted in an increase in the absorption at 2750 Å and a decrease at 2500 Å. When insulin was irradiated for a short time, the increase in the absorption at the maximum was slightly greater than that at the minimum. As irradiation proceeded, the absorption at the minimum increased more rapidly, and the absorption curve began to flatten out. The activity of the hormone was destroyed after a short period of irradiation, but the absorption spectrum changed more slowly. Apparently more than one reaction occurred, but not all reactions resulted in inactivation.

Insulin has also been irradiated in monolayers[13, 14], and free tyrosine has been liberated into the bulk of the solution. Partial liquefaction of the gel, which has been observed[13] upon irradiation of the monolayer film, has been inhibited by hydroquinone.

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Phenol has also been found[6, 10] to inhibit the inactivation of insulin by ultraviolet light. These facts have been interpreted as evidence for oxidative fragmentation of insulin upon irradiation. However, it is also possible that the protective action of the phenols is a result of their preferential absorption of ultraviolet light.

KUHN, EYER, AND FREUDENBERG[9] also observed that during the irradiation of insulin or of a mixture of tyrosine and cystine, free ammonia and hydrogen sulphide were formed and the presence of aldehydes could be detected, especially in the later stages of the reaction. When insulin reacted with formaldehyde, *o*-chlorobenzaldehyde, or hydrogen peroxide, its absorption spectrum was modified similarly to that of the irradiated hormone. It was suggested that aldehydes are formed upon irradiation of insulin and then react with active portions of the protein molecule. Even if such a reaction did occur, there was no evidence that it was responsible for the inactivation of the hormone, since aldehydes were detected only after the biological activity had disappeared.

In the present investigation solutions of amorphous insulin of the same concentration have been irradiated with light of wavelength 2537 Å for various periods of time, and the activities of the irradiated solutions have been determined. The loss of activity has been found to be first-order with respect to the amount of light absorbed up to a 60% loss in activity, and the average quantum yield was 0.015 moles per einstein. The absorption spectrum of partially inactivated insulin was compared with that of the unirradiated sample. Since a sample of crystalline zinc-insulin was available, a quantum yield was also determined for this material. The quantum yield for the inactivation of the crystalline zinc-insulin was somewhat lower (0.009) than for the amorphous insulin, but in view of the variation in the experimental points, the difference can be considered negligible. Since there was evidence that certain chemical changes occurred upon irradiation of insulin solutions, an attempt was made to correlate these changes with changes in the biological activity. Since it has become necessary to abandon this investigation, the results obtained are now being reported.

EXPERIMENTAL

Quantum Yield for the Inactivation of Amorphous Insulin

The insulin used was a sample of amorphous insulin obtained by isoelectric precipitation. Electrophoresis indicated the presence of about 5% protein impurity[16]. By drying over phosphorus pentoxide the solid protein was found to contain 7.0% moisture.

In each case solid insulin (29.1 mg) was dissolved in 25.00 ml 0.005 M acetate buffer, p_H 4.3, to give a solution of p_H 4.0 containing 1.16 mg per ml of the protein as weighed. Allowing for the moisture and the protein impurity, the insulin concentration was approximately 1.03 mg per ml. The variation in light absorption at 2537 Å indicated that the solution was not exactly reproducible by weighing out the solid protein and diluting in a volumetric flask. The exact protein concentration was therefore calculated by means of BEER's law. The optical density at 2537 Å was determined with a Beckman spectrophotometer and compared with that of a solution of known concentration of the dehydrated protein. The calculated concentrations are given in Table I.

Samples of the protein solution were irradiated at 2537 Å in the apparatus previously described[17]. The irradiated samples were compared with unirradiated controls by the mouse convulsion method[16] of TREVAN AND BOOCK as described in the 1926 *Report of*

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the League of Nations. The controls were assumed in each case to have an insulin activity of 25 units per ml. The controls and the irradiated samples were diluted to that concentration at which about 50% of 30 mice had convulsions upon injection. The activities were assumed equal at these dilutions, and the activities of the undiluted irradiated samples could be estimated. The assays are believed to be accurate within about 5% and the results are indicated in Table I.

Quantum yields were calculated using the equation $\Phi = \frac{2.303\, E_o \log E_o/E}{I\, f\, t}$, in

TABLE I

INACTIVATION OF AMORPHOUS INSULIN BY IRRADIATION AT 2537 Å

Experiment	Insulin concentration mg/ml	% of light absorbed before Irradiation at λ 2537 Å	Intensity of light einsteins per ml per hour	Irradiation time hours	Units activity remaining	% of original activity remaining	log %	log E_o/E	Φ moles per einstein
1	1.04	69.8	$0.162 \cdot 10^{-4}$	0.033	19.75	79.0	1.898	0.102	0.018
2	0.942	66.0	$0.156 \cdot 10^{-4}$	0.033	18	72.0	1.857	0.143	0.025*
3	1.04	69.8	$0.162 \cdot 10^{-4}$	0.058	18.75	75.0	1.875	0.125	0.013
4	1.07	70.7	$0.155 \cdot 10^{-4}$	0.083	15	60.0	1.778	0.222	0.017
5	1.04	69.8	$0.162 \cdot 10^{-4}$	0.116	13.1	52.4	1.719	0.281	0.014
6	1.07	70.7	$0.155 \cdot 10^{-4}$	0.167	10.5	42.0	1.623	0.377	0.014
7	0.942	66.0	$0.156 \cdot 10^{-4}$	1.00	2.7	10.8	1.033	0.967	0.006*
								Average	0.015

* Not included in the average.

which Φ is the quantum yield in moles per einstein, E_o the initial insulin concentration in moles per ml, E the concentration after irradiation, I the intensity of the light falling on the solution in the quartz cell in einsteins per ml per hour, f the fraction of light of λ 2537 Å initially absorbed by the protein solution, and t the time of irradiation in hours[18]. It was assumed that all the light absorbed by the protein solution initially was absorbed by active insulin. If this was not the case, the quantum yields may be slightly higher than indicated in Table I. The molecular weight of insulin was taken as 36000. Recent publications[19, 20, 21] have indicated that the molecular weight of insulin may be as high as 48000. However, in dilute solution (< 0.3%) and at p_H's below 4.0 and above 7.5, the molecule is evidently dissociated to particles of molecular weight 12000[20, 21, 22, 23]. Since the extent of the dissociation of insulin in the solutions irradiated was unknown, the quantum yields may be three-fourths to three times as great as those given in Table I.

The variation of activity remaining with the time of irradiation is shown in Fig. 1 to follow the law for a first-order reaction up to 10 minutes of irradiation, at which the activity has been reduced to 40% of that of the unirradiated solution. The point obtained in experiment 2 deviated somewhat from the curve, so it was omitted in calculating the average quantum yield of 0.015 moles per einstein. The average deviation was 0.002. Experiment 7 was also not included in the average, as the occurrence of secondary reactions, and increased light absorption may have been responsible for the low quantum yield at that stage.

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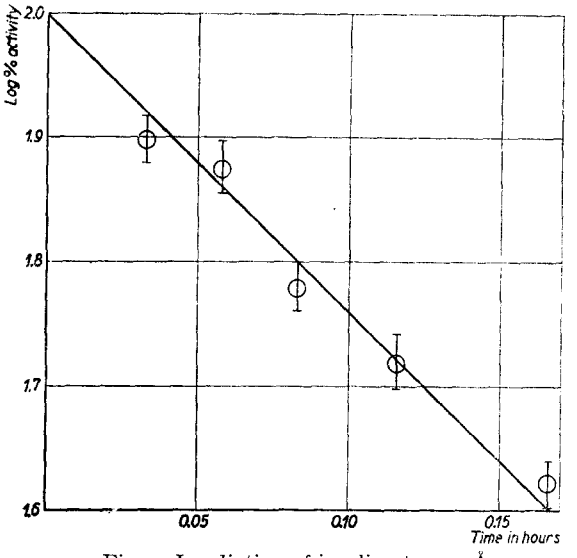

Fig. 1. Irradiation of insulin at 2537 Å

Absorption Spectrum of Irradiated Insulin

Samples of the insulin solution were irradiated 2.5, 5, and 10 minutes, respectively. The irradiated solutions and a sample of the unirradiated insulin solution were diluted with an equal volume of buffer. The absorption spectra of the dilute solutions were determined by means of a Beckman D. U. ultraviolet quartz spectrophotometer. In Fig. 2 optical density is plotted against wave length for each of the solutions. As expected from the data in the literature[9], the optical density at any wavelength was greatest for the solution which had been irradiated longest. It increased approximately linearly with the time of irradiation. The molecular extinction coefficients at the maximum (2765–2770 Å) and the minimum (2500 Å) have been calculated, using 36000 as the molecular weight of insulin, and are shown in Table II.

According to BRAND[24] insulin contains 7.9% phenylalanine, 12.3% tyrosine, 11.0% cystine, and no tryptophane. On the basis of a molecular weight of 36000, these figures would correspond to 17 phenylalanine, 24 tyrosine, and 33 (½-cystine) residues per molecule of insulin. Values of the extinction coefficients of cystine were calculated from the data of FOSTER, ANSLOW, AND BARNES[25] and of phenylalanine and tyrosine from the data of LERNER AND BARNUM[26]. The contribution of phenylalanine was very small compared with the other two amino acids, and the total extinction coefficients for these amino acids in the quantities present in insulin were 21000 at the maximum and 12000 at the minimum. The experimental values for unirradiated insulin were approximately double the calculated extinction coefficients. The differences may result from the small contributions of peptide bonds

TABLE II

EFFECT OF IRRADIATION UPON EXTINCTION COEFFICIENTS

Irradiation time min	Maximum extinction coefficient	Minimum extinction coefficient
0	$4.27 \cdot 10^4$	$2.06 \cdot 10^4$
2.5	$4.72 \cdot 10^4$	$2.28 \cdot 10^4$
5	$5.10 \cdot 10^4$	$2.51 \cdot 10^4$
10	$5.84 \cdot 10^4$	$3.04 \cdot 10^4$

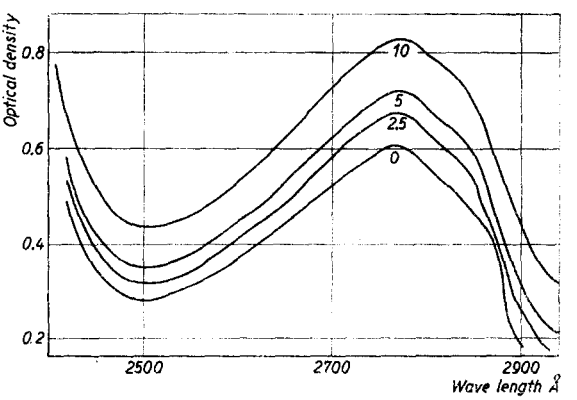

Fig. 2. Effect of irradiation on the absorption spectrum of insulin. Figures give time of irradiation in minutes.

or of other amino acids in the protein molecule, the influence of other groups in the protein on the absorption by cystine and tyrosine, or the effect of p_H.

Quantum Yield for Inactivation of Crystalline Zinc Insulin

The amorphous insulin had a purity of only about 95%, and since a small quantity of crystalline zinc-insulin was available, it was desired to compare the quantum yields for the inactivation of the two preparations by light of wavelength 2537 Å. Crystalline zinc-insulin (26.0 mg) was dissolved in 26.00 ml 0.0086 M acetate buffer, p_H 3.9. A clear solution was obtained only after the mixture had been standing over night in the refrigerator, and the solution had a p_H of 4.1. The zinc-insulin had a moisture content of 3.5%, and as in the case of amorphous insulin, exact concentrations in solution were estimated by comparison of optical densities at 2537 Å with the optical density of a solution containing a known quantity of the anhydrous crystalline zinc-insulin. The calculated concentrations, activities of the irradiated solutions, and quantum yields are given in Table III. The activity of the unirradiated solution was taken as 22.0 units. The mean quantum yield was 0.009 moles per einstein with an average deviation of 0.003. This value is somewhat lower than that for the amorphous insulin, but the difference is negligible in view if the experimental variation.

TABLE III

INACTIVATION OF CRYSTALLINE ZINC-INSULIN BY IRRADIATION AT 2537 Å

Experiment	Insulin concentration mg/ml	% of light absorbed before irradiation at λ 2537 Å	Intensity of light einsteins per ml per hour	Irradiation time hours	Units activity remaining	% of original activity remaining	log %	log E_o/E	Φ moles per einstein
8	1.05	72.3	$0.150 \cdot 10^{-4}$	0.042	20.0	90.9	1.959	0.041	0.006
9	1.05	72.3	$0.150 \cdot 10^{-4}$	0.083	18.2	82.6	1.917	0.083	0.006
10	0.977	70.0	$0.158 \cdot 10^{-4}$	0.125	12.9	58.7	1.769	0.231	0.010
11	1.05	72.3	$0.150 \cdot 10^{-4}$	0.167	12.4	56.4	1.751	0.249	0.009
12	0.977	70.0	$0.158 \cdot 10^{-4}$	0.208	7.7	35.0	1.544	0.456	0.012
13	1.05	72.3	$0.150 \cdot 10^{-4}$	0.250	7.8	35.4	1.549	0.451	0.011
								Average	0.009

Chemical Changes Upon Irradiation of Insulin

CARPENTER[14] and MITCHELL AND RIDEAL[13] found that free tyrosine is liberated into the bulk of the solution when insulin monolayers are irradiated. The liberation of free tyrosine by irradiation of an insulin solution has been confirmed qualitatively by paper chromatography*. A quantitative investigation[27] of the liberation of tyrosine by irradiation of 0.2% insulin solutions indicated that the liberation of tyrosine is linear with respect to time of irradiation up to 30 minutes of irradiation at which point the inactivation, for a light intensity of $0.15 \cdot 10^{-4}$ einsteins per hour as calculated from the quantum yield, would be about 99%. Upon further irradiation the rate of liberation of tyrosine increased with time. The quantum yield for the liberation of tyrosine upon 15 minutes irradiation was only 0.002 compared with 0.015 for the inactivation of insulin.

* The authors are indepted to Mr N. LEITNER of the *Polytechnic Institute of Brooklyn* for this determination.

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The liberation of free ammonia upon irradiation of insulin has also been confirmed[27], but it does not correspond to the loss of biological activity. Ammonia was not detected upon irradiation of a 0.2% solution for 75 minutes, but was only detected after 3 hours of irradiation, when there was no longer any hormone activity.

After the insulin solutions had been irradiated, the odour of burning hair could be detected. This was an indication that some change in the cystine must have occurred, especially in view of the observations of KUHN, EYER, AND FREUDENBERG[9] on the changes in ultraviolet absorption characteristics upon irradiation of cystine and of insulin. No free cystine or cysteine has been found after irradiation of insulin[27]. MIRSKY AND ANSON[28] reduced the native and denatured forms of proteins containing cystine by means of thioglycolic acid. The denatured forms than gave a quantitative test for groups capable of reducing cystine whereas the native forms did not. GREENSTEIN[29] found that native insulin did not reveal the presence of disulfide groups by the cyanide-nitroprusside test, whereas samples denatured with urea or guanidine hydrochloride gave positive test for disulfide groups. The denatured insulin preparations were biologically active in this case. In the present investigation irradiated and unirradiated samples of insulin were reduced with thioglycolic acid and tested for reducing groups by the method of MIRSKY AND ANSON[28]. No difference has been detected between native insulin and insulin irradiated to reduce its activity to 53% of the original. Both the reduced native and the reduced irradiated protein reduced cystine to a slight extent, but this may be a result of failure to wash out the excess thioglycolic acid completely.

Since tyrosine was liberated, it was thought that some information might be obtained with the ultracentrifuge as to whether only groups at the end of the peptide chain were attacked or whether groups within the chain reacted as well. A 1.0% solution in 0.01 M acetate buffer at p_H 3.4 was irradiated and the irradiated solution and an unirradiated control were sedimented* in the ultracentrifuge at 200000 g. In both samples the bulk of the protein was apparently dissociated, so that no appreciable sedimentation occurred at this force, and no differences could be detected.

DISCUSSION

The quantum yield for the inactivation of amorphous insulin at 2537 Å has been shown to be 0.015. The quantum yield for the inactivation of crystalline zinc-insulin is 0.009. In view of the variation in the experimental points, this difference is very small. It may, however, be a result of protective action of the zinc on the insulin. It would be desirable to investigate the effect of zinc during the irradiation of amorphous insulin.

The liberation of tyrosine upon irradiation of insulin is insufficient to account for the loss of biological activity, even if loss of only one molecule of tyrosine from a molecule of insulin will inactivate that molecule. The inactivation of insulin is not related to liberation of ammonia, as no detectable quantities of ammonia are formed until after the hormone has been completely inactivated. No free cysteine or cystine is formed by irradiation of insulin. The change in the absorption spectra upon irradiation of solutions of insulin, cystine, tyrosine, and mixtures of cystine and tyrosine would indicate that the initial reaction occurring upon irradiation of insulin largely involves the cystine,

* The authors are grateful to Mr E. SHEPPARD of the *Polytechnic Institute of Brooklyn* for this determination.

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the tyrosine becoming involved to a greater extent as the reaction proceeds. The appearance of the odour of burning hair after a short period of irradiation would also indicate that cystine is involved in the initial reaction. Apparently, irradiation of insulin does not result in a change in the reducing power of the reduced protein for cystine, by the method of MIRSKY AND ANSON[28]. Although it is possible that oxidation occurs during the irradiation of insulin, it is unlikely that atmospheric oxidation occurs, since inactivation has been shown[6] to occur in the absence of air or oxygen. Recently, it has been shown[18] that chymotrypsin gives the same quantum yield when irradiated either in an atmosphere of oxygen or of nitrogen. It may be possible on further investigation to isolate some oxidation product of cystine from irradiated insulin solutions. Since under the conditions of the experiment described, insulin did not sediment in the ultracentrifuge, it would also be desirable to investigate the effect of irradiation on the osmotic behaviour of insulin in order to determine whether there is complete fragmentation of the peptide chain or removal of small fragments from the ends of the chain.

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SUMMARY

1. The quantum yield for the inactivation of insulin at wave length 2537 Å has been found to be 0.015. Inactivation proceeds by a first order process.

2. Accompanying a loss in activity there is an increase in the ultraviolet absorption spectrum of insulin. This increase is probably associated with photolysis of cystine and tyrosine residues with the molecule.

3. The liberation of tyrosine from the molecule does not parallel the loss of biological activity. The inactivation of insulin precedes any liberation of ammonia.

RÉSUMÉ

1. Nous avons trouvé pour l'inactivation de l'insuline par de la lumière d'une longueur d'onde de 2537 Å un rendement de 0.012 mols par einstein. L'inactivation procède selon une réaction de premier ordre.

2. La perte d'activité est accompagnée d'une augmentation de l'absorption dans l'ultraviolet. Cette augmentation est probablement associée à la photolyse de restes de cystine et de tyrosine de la molécule.

3. La mise en liberté de tyrosine à partir de la molécule ne se produit pas parallèlement à la perte d'activité. L'inactivation de l'insuline précède toute mise en liberté d'ammoniac.

ZUSAMMENFASSUNG

1. Für die Desaktivierung von Insulin bei einer Wellenlänge von 2537 Å wurde eine Ausbeute von 0.012 Mol. per Einstein gefunden. Die Desaktivierung verläuft wie eine Reaktion erster Ordnung.

2. Die Abnahme der Aktivität ist von einer Zunahme der Ultraviolettabsorption des Insulins begleitet. Diese Zunahme ist wahrscheinlich auf die Photolyse von Cystin- und Tyrosinresten in der Molekel zurückzuführen.

3. Das Freiwerden von Tyrosin aus der Molekel verläuft nicht parallel mit dem Aktivitätsverlust. Das Insulin wird desaktiviert bevor Ammoniak abgegeben wird.

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