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Photooxidation of Bovine Insulin Sensitized by Methylene Blue

LEOPOLD WEIL,¹ THOMAS S. SEIBLES, AND THEODORE T. HERSKOVITS

Eastern Regional Research Laboratory,² Philadelphia, Pennsylvania

Photooxidation of insulin at pH 7.0 and at 10° was confined solely to the oxidation of the two histidine residues of the hormone. Photooxidation of insulin above 10° brought about an additional and progressive involvement of the tyrosine residues.

The rate and extent of photooxidation of insulin is markedly influenced by the conformation of the molecule.

A correlation has been established between photooxidation of the two histidine residues of insulin and its biological activity.

The effect of photooxidation on the solvent perturbation difference spectra and on the rotatory dispersion parameters indicate that the observed inactivation of insulin was not due to changes in the secondary and tertiary structure of the hormone.

Zinc-free insulin appears to have a somewhat more unfolded structure than Zn-insulin.

Although the primary structure of insulin was completely elucidated by Sanger *et al.* some years ago (1-3), the correlation of its structure with physiological activity is not definitely established. The difficulties in demonstrating such an interrelationship are partly due to the insulin assay procedure itself, which requires either the intact animal or the intact tissue—a rather complex system for establishing precisely the specific involvement of this hormone. In addition, insulin exerts its action not only on carbohydrate metabolism but also is concerned with metabolism of fats and proteins. Whether this multiplicity of action is due to a single process remains to be answered. A detailed discussion of various aspects of these problems has been presented recently by Young (4). Our present work deals with the photoinactivation of insulin by sensitized visible light and with the possible involvement of the imidazole side-chains of this hormone in its physiological activity.

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² Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

EXPERIMENTAL

Materials

Crystalline Zn-insulin used throughout this work was supplied by Dr. O. K. Behrens of the Lilly Co. Because of the limited solubility of this preparation at pH 7.0, the insulin was freed of Zn by the method of Sluyterman (5) and used as such.

Biological assay. The biological assays were carried out through the courtesy of Dr. R. M. Ellis of the Lilly Research Laboratories; the mouse convulsion technique was used. Ten 30-mouse assays were done for each sample. The potency of the starting Zn-free preparation was 23.6 U per milligram.

Irradiation procedure. The method used was that described in our previous work (6, 7) but at a temperature of 10°. In general, the main chamber of the Warburg vessel was charged with 1 ml Zn-free insulin (10 mg) adjusted to pH 7.0 and with 0.5 ml of 0.1 M phosphate buffer of pH 7.0. In the sidearm of the vessel was placed 0.5 ml of Zn-free methylene blue solution (0.1 mg), and the center well was charged with 0.2 ml of 20% of KOH together with a cylindrical piece of filter paper to absorb any CO₂ which might evolve during the photooxidation. After temperature equilibrium at 10°, the system was closed, the methylene blue solution was tipped into the main chamber, and the irradiation was started.

RESULTS

Effect of photooxidation of insulin at 10° on its amino acid composition. Zinc-free insulin was photooxidized at pH 7.0 and at 10°, as described above, to various degrees of oxygen uptake. Calculations were based on a molecular weight of 5734. After the desired oxidation level was obtained, the respective sample was transferred, treated with a small amount of purified charcoal to remove the dye, and filtered through a small sintered glass disc, and the filtrate was brought to dryness with a rotatory evaporator. The dry residue was then heated with 2 ml of 6 N HCl at 120° for 24 hours in an evacuated sealed tube. The hydrolyzate was evaporated to dryness, and after dissolving the residue in 10 ml of 0.2 M citrate buffer of pH 2.2, aliquots were taken for amino acid analysis (8). The results obtained and presented in Table I indicate that, under the conditions employed, only the histidine content of insulin was affected by photooxidation. The amino acid analysis as a whole agrees well with values reported in the literature (9) except for the somewhat lower cystine and tyrosine values, which are not unexpected in view of the tendency of these

amino acids to undergo some destruction during acid hydrolysis.

Changes in ultraviolet spectrum of insulin during photooxidation. The photooxidation of insulin was performed as described before. After removal of the dye by charcoal, the ultraviolet spectra were measured at appropriate dilutions. The loss of protein was checked by total nitrogen determination and all measurements were corrected accordingly. The results obtained (Fig. 1) indicate that the observed changes in the spectrum were due solely to the photooxidation of the two histidine residues of the B-chain of insulin. The molar extinction coefficient, ϵ_M , of native insulin (Curve *a*) at 278 m μ increased from 6080 to 7225 at 1 mole of oxygen uptake per mole of insulin (Curve *b*), while at 2 moles of oxygen uptake (Curve *c*) this value rose to 8430. This increase in extinction coefficient was due to the increased absorption of photooxidized histidine, as indicated by Curve *d*, which represents the contribution of the photooxidized histidine alone to the absorption spectrum of insulin. Thus, by subtracting from the ϵ_M of 8430, which was obtained after 2 moles of oxygen uptake per mole o-

TABLE I
CHANGES IN AMINO ACID COMPOSITION DURING THE PHOTOOXIDATION OF INSULIN AT pH 7.0 AND AT 10° AFTER 24 HOURS HYDROLYSIS

Amino acids	% Amino Acids					
	Moles of O ₂ per mole of insulin					
	0.0	0.48	0.99	1.48	2.01	2.25
Lysine	2.54	2.41	2.50	2.47	2.51	2.50
Histidine	5.41	3.92	2.70	1.90	0.95	0.10
Arginine	3.10	2.99	2.98	3.08	3.07	3.03
Aspartic acid	6.84	6.74	6.78	6.93	6.98	6.86
Threonine	2.08	2.14	2.18	2.09	2.12	2.15
Serine	4.99	4.91	4.99	5.14	5.17	5.18
Glutamic acid	17.70	17.42	17.48	17.62	17.51	17.48
Proline	2.14	2.17	2.05	2.11	1.99	2.16
Glycine	5.35	5.20	5.17	5.32	5.28	5.15
Alanine	4.66	4.52	4.48	4.60	4.56	4.50
½ Cystine	10.55	10.08	10.67	10.67	10.48	10.43
Valine	8.94	8.69	8.73	9.04	8.96	8.84
Isoleucine	1.48	1.58	1.39	1.43	1.42	1.38
Leucine	12.62	12.56	12.32	12.63	12.38	12.37
Tyrosine	10.02	9.94	9.98	9.94	9.80	9.98
Phenylalanine	8.28	8.14	8.04	8.20	8.08	8.12

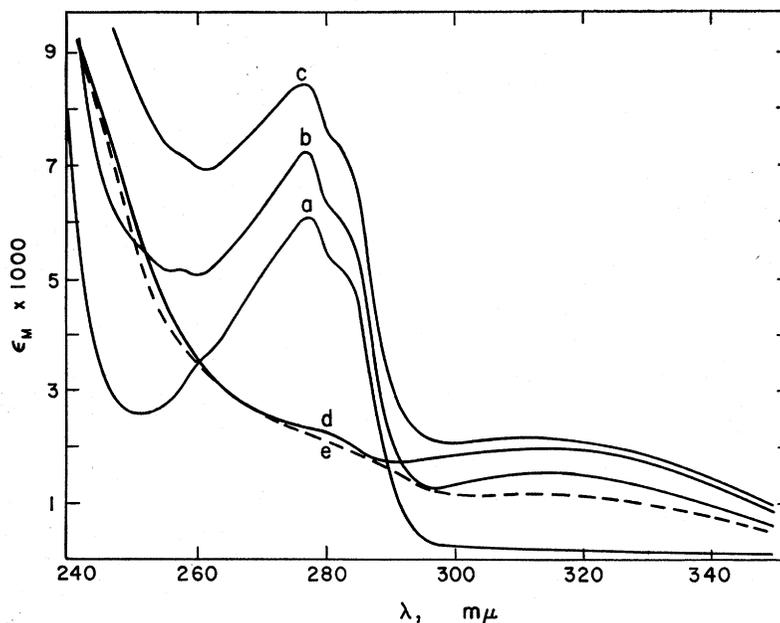


FIG. 1. Changes in the ultraviolet absorption spectrum of insulin produced by photooxidation. *a*, Native insulin; *b*, 1 mole of O₂ per mole of insulin; *c*, 2 moles of O₂ per mole of insulin; *d* = *c* - *a*; *e*, ultraviolet spectrum of photooxidized free histidine. Buffer, 0.025 *M* phosphate, pH 7.0.

insulin, the ϵ_M value of 2295, which is due to the absorption of 2 moles of photooxidized histidine at this wavelength (278 $m\mu$), one obtains an ϵ_M value of 6135, which is in good agreement with the value of 6080 obtained with the native insulin. The difference spectrum obtained (Curve *d*) by subtracting Curve *a* from Curve *c* resembles Curve *e* quite well, and supports the fact that the observed changes in ultraviolet spectrum of insulin during photooxidation were due solely to histidine. Deviation in Curves *e* and *d* beyond 290 $m\mu$ might be due to the differences between spectral changes of photooxidized-free and peptide-bonded histidine.

Effect of temperature on the photooxidation of insulin at pH 7.0. Experimental details were identical with those indicated above, and the temperature was maintained as described previously (7). The rates of oxygen uptake obtained at 2°, 10°, 20°, and 40° (Fig. 2) show that at 2° and 10° the photooxidation of insulin is confined to 2.3 moles of oxygen per mole of insulin, at which point, the photooxidation is limited solely

to 2 moles of histidine (Table I). The slightly higher oxygen uptake over the 2 moles required theoretically is due to a "slow" reaction. The progressive increase in rates and extent of oxygen uptake at 20° and 40° is very probably due to photooxidation of tyrosine residues at these elevated temperatures (7).

Effect of 8 M urea on the photooxidation of insulin and the A- and B-chains of insulin at pH 7.0 and at 10°. To study the possible effect of the conformation of insulin on the rate and extent of photooxidation, the influence of urea on this reaction was studied, and the results were compared with those obtained in water solution. Similar studies were carried out with the A- and B-chains derived from insulin according to the procedure of Mycek *et al.* (10). In addition, parallel photooxidation studies were made with model amino acid mixtures composed of the photoreactive amino acids (histidine and tyrosine) in amounts equivalent to those in which they occur in the protein. These experiments were designed to demonstrate the maximum rate of photooxidation on the

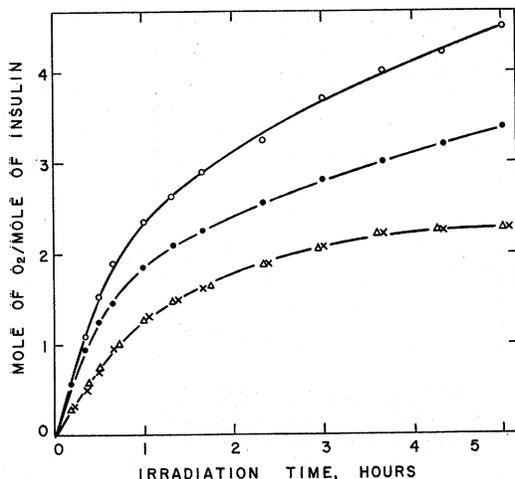


FIG. 2. Effect of temperature on the photo-oxidation of insulin in 0.025 *M* phosphate, pH 7.0. \times — \times , at 2°; \triangle — \triangle , at 10°; \bullet — \bullet , at 20°; \circ — \circ at 40°. Protein concentration, 0.5%.

assumption that the rate of photooxidation of the free photoreactive amino acids is the same as when they were present in the substrate in the fully exposed form.

For experiments carried out in aqueous solution, to 1.5 ml of solution containing 1.74 μ moles of insulin, A- or B-chain of insulin in 0.033 *M* phosphate buffer, pH 7.0, respectively, and 0.5 ml of methylene blue (0.1 mg) were added, and the solution was irradiated as described before. In experiments carried out in urea solution, the composition of the reaction mixture was the same as above except that 8 *M* urea was used as a solvent both for the substrate and methylene blue. In model experiments, a solution equivalent to the photoreactive amino acids present in 1.74 μ moles of insulin contained 3.49 μ moles of histidine plus 6.98 μ moles of tyrosine, a solution equivalent to 1.74 μ moles of A-chain of insulin contained 3.49 μ moles of tyrosine, while a solution equivalent to 1.74 μ moles of B-chain of insulin contained 3.49 μ moles of histidine and 3.49 μ moles of tyrosine per 1.5 ml of 8 *M* urea solution (in 0.033 *M* phosphate buffer of pH 7.0). The pH values of all solutions used were adjusted to pH 7.0. Irradiation of 8 *M* urea in the presence of methylene blue was not accompanied by

any oxygen uptake. The results obtained (Fig. 3) indicate that the rate and extent of photooxidation of insulin depends on the conformation of the molecule. In the native state (Fig. 3A) photooxidation was confined to about 2.3 moles of oxygen uptake per mole of insulin, at which point (Table I) only the two histidine residues of insulin were photooxidized. In an identical experiment with 8 *M* urea as solvent there was not only an increased rate of histidine oxidation but also unmasking and photooxidation of tyrosine, which in the native state was unavailable at this temperature. The rate of photooxidation of the model amino acid mixture in 8 *M* urea was identical to that obtained with insulin in the same solvent up to 2 moles of oxygen per mole of insulin (at which point only the two histidine residues have reacted), but above that level the rate with the amino acid mixture was higher than that obtained with insulin. This latter observation might be indicative that insulin retains a certain degree of structure even in 8 *M* urea solution, or that the fully exposed but protein-bonded tyrosine residues have a somewhat lower rate of oxidation than the free amino acid.

Photooxidation in aqueous solution of the A-chain of insulin (Fig. 3B), which contains only 2 tyrosine residues, as expected resulted only in a very slight oxygen uptake which was only very moderately increased if the experiment was repeated in 8 *M* urea solution. On the other hand, photooxidation of the model amino acid solution corresponding to the A-chain produced a marked oxygen uptake.

Photooxidation of the B-chain of insulin (Fig. 3C), which contains 2 histidine and 2 tyrosine residues, in water solution resulted in a somewhat accelerated rate and extent of oxygen uptake over that observed for insulin (Fig. 3A), and this became more pronounced in 8 *M* urea solution. Photooxidation of the model amino acid mixture corresponding to the B-chain of insulin, in this solvent, produced an identical rate of oxygen uptake up to 2 moles of oxygen, as observed for the B-chain in the same solvent; but above this level the rate of oxygen uptake was found to be higher. Figure 3D represents

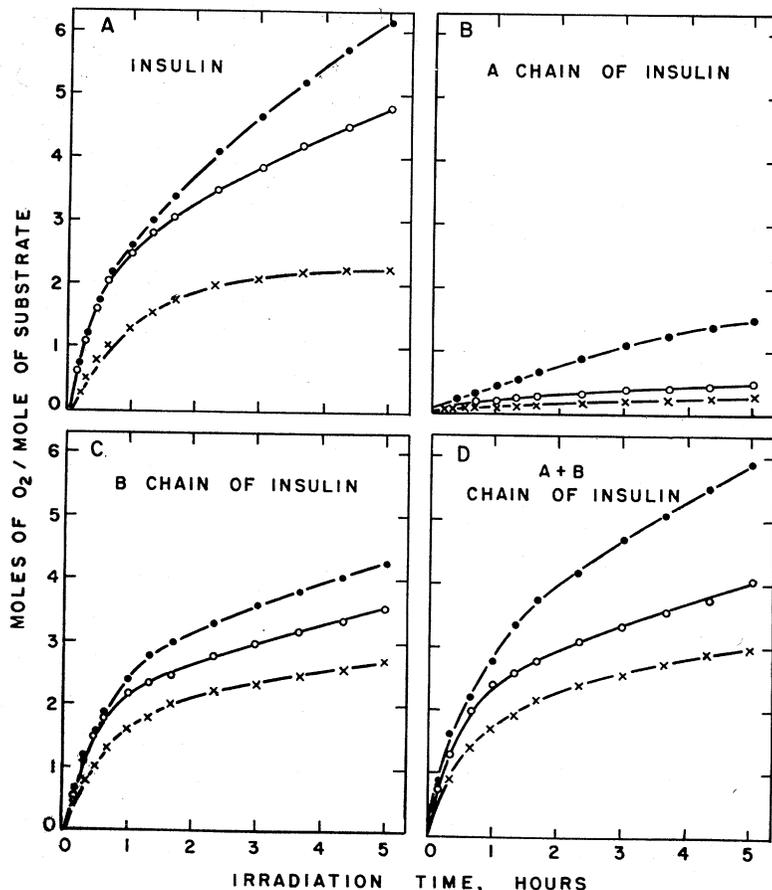


FIG. 3. Photooxidation of insulin, A- and B-chain of insulin in 0.025 *M* phosphate, pH 7.0 and at 10°. ×—×, Substrate in water; ○—○, substrate in 8 *M* urea; ●—●, model amino acid mixture in 8 *M* urea. Concentration of insulin, A- and B-chains of insulin, 1.31×10^{-3} *M*, respectively. The model amino acid mixtures had the following concentration: for insulin, 2.62×10^{-3} *M* histidine, 5.24×10^{-3} *M* tyrosine; for the A-chain, 2.62×10^{-3} *M* tyrosine; for the B-chain, 2.62×10^{-3} *M* histidine, 2.62×10^{-3} *M* tyrosine.

the sum of the oxygen uptakes for the three curves presented in Fig. 3B and 3C.

To show that the increased reactivity of the model amino acid mixtures (Fig. 3) in 8 *M* urea solution observed in comparison with the parent protein was not due to any solvent effect, the rates and extents of photooxidation of the four photoreactive amino acids (histidine, tyrosine, methionine, and tryptophan) (7) were compared in water and 8 *M* urea solution. The results obtained (Fig. 4) demonstrate that the rates and extent of photooxidation of these amino acids were the same whether water or 8 *M* urea was the solvent.

Photooxidation of Zn-insulin and Zn-free insulin at pH 8.0 and at 10°. These experiments were carried out at pH 8.0 because of the limited solubility of Zn-insulin at pH 7.0. Irradiation experiments were carried out as before. The reaction mixture consisted of 1 ml of 1% insulin solution at pH 8.0, 0.5 ml of 0.1 *M* phosphate buffer of pH 8.0, and 0.5 ml of Zn-free methylene blue solution (0.1 mg). The results in Fig. 5 indicate that Zn-insulin was photooxidized at a distinctly lower rate than the Zn-free preparation. The higher level of oxygen uptake observed at pH 8.0 over that obtained at pH 7.0 (Fig. 3) is due to the involvement

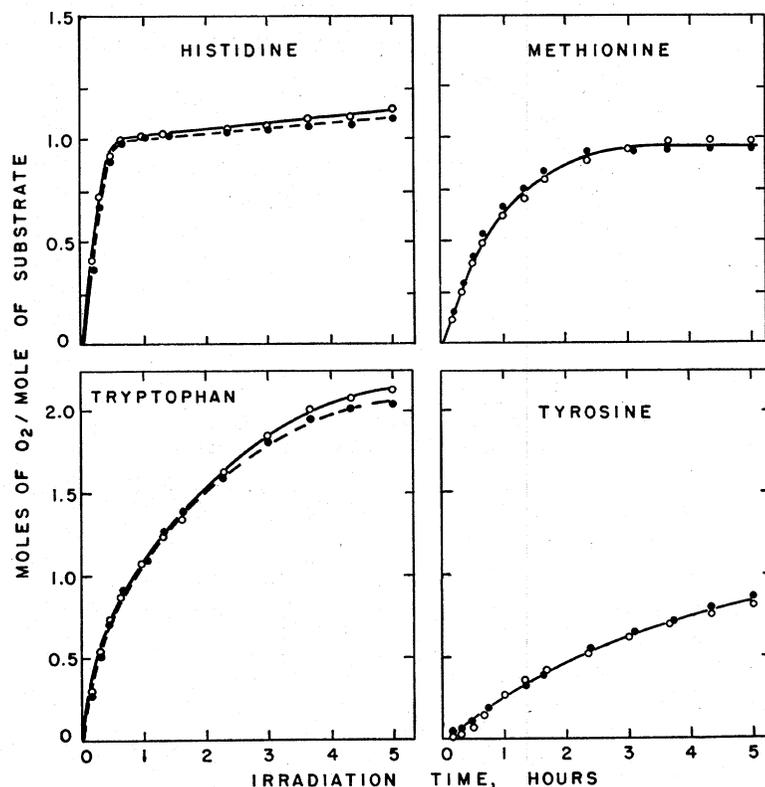


FIG. 4. Photooxidation of histidine, methionine, tryptophan, and tyrosine in water and 8 *M* urea in 0.025 *M* phosphate, pH 7.0, and at 10°. ○—○, In water; ●—●, in 8 *M* urea. Concentrations: histidine and methionine, 3.75×10^{-3} *M*; tyrosine and tryptophan, 1.88×10^{-3} *M*.

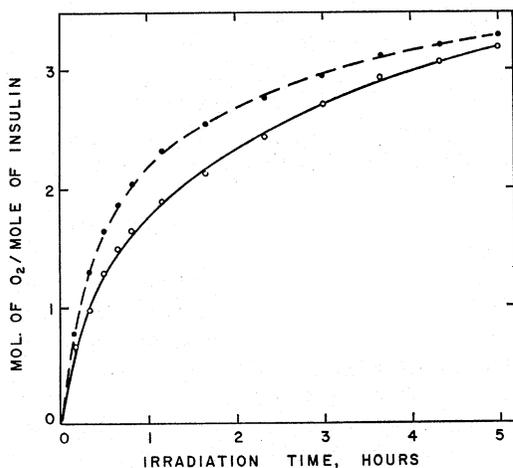


FIG. 5. Photooxidation of Zn-insulin and Zn-free insulin in 0.025 *M* phosphate, pH 8.0, and at 10°. ○—○, Zn-insulin; ●—●, Zn-free insulin. Protein concentration, 0.5%.

of tyrosine residues in the photooxidation (7). Since the lower rate of photooxidation of Zn-insulin (Fig. 5) might be due to complex formation between Zn^{++} and the imidazole side-chains of insulin, the photooxidation of free histidine was compared in the absence and presence of Zn^{++} . One reaction mixture consisted of 1.5 ml of a solution of 5 μ moles of histidine at pH 7.0 plus 0.5 ml of Zn-free methylene blue (0.1 mg); the second reaction mixture was the same except that it contained an additional 5 μ moles of $ZnSO_4$. Irradiation was carried out at 10°. The results in Fig. 6 show that addition of Zn had no inhibitory effect on the photooxidation.

Correlation between insulin activity and photooxidation of histidine in insulin. Zinc-free insulin was photooxidized at pH 7.0 and 10° as described for the results presented

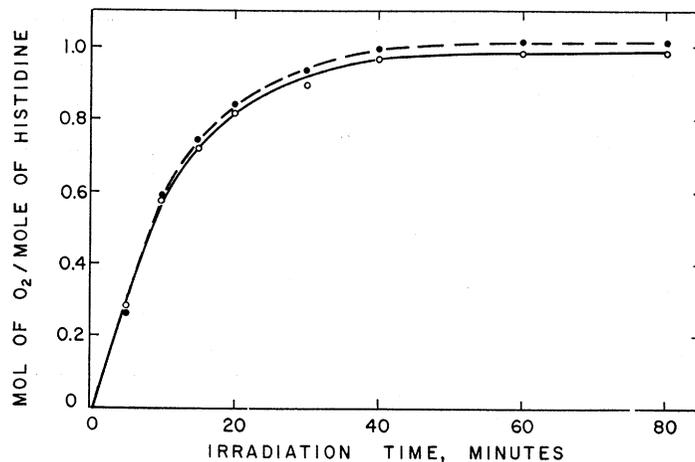


FIG. 6. Photooxidation of histidine in the presence and absence of Zn^{++} in $0.025 M$ phosphate, pH 7.0, and at 10° . \circ — \circ , Histidine; \bullet — \bullet , histidine + Zn^{++} ($3.75 \times 10^{-3} M$). Histidine concentration, $3.75 \times 10^{-3} M$.

in Table I. The small quantities of methylene blue added in photooxidation were not removed prior to the biological assays. Preliminary tests indicated that the quantities involved did not interfere. Control samples irradiated without the dye exhibited no impairment of biological activity. The results presented in Fig. 7, in which the percentage of insulin activity remaining is plotted against moles of histidine photooxidized, clearly indicate a close correlation between biological activity and histidine content.

Figure 8 is a semilogarithmic plot of the loss of biological activity and extent of photooxidation of histidine in insulin as a function of time. Destruction of biological activity and histidine follow identical first-order kinetics, with a rate constant of 0.0182 min^{-1} . Photooxidation of an equivalent amount of free histidine (Fig. 8) proceeds at a much faster rate, and essentially in a zero-order fashion. This suggests that the rate of photooxidation of histidine within the insulin molecule is a function of the three-dimensional structure of the molecule (cf. Fig. 3).

Histidine-containing peptides isolated from native and photooxidized insulin after peptic digestion. Although the results in Fig. 8 indicate that both the histidine residues located in positions 5 and 10 of the B-chain

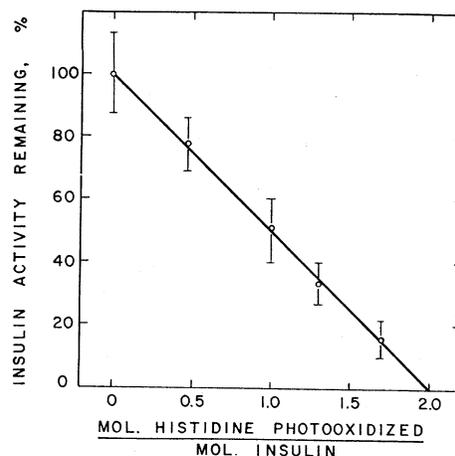


FIG. 7. Correlation between photooxidation of histidine in insulin and biological activity.

were photooxidized at the same rate, experiments were carried out to verify this assumption by isolating the histidine-containing peptides of insulin before and after 1 mole of oxygen uptake per mole of protein. The photooxidation was carried out as described before at pH 7.0 and at 10° . Three hundred mg of native, unreacted insulin or insulin photooxidized to 1 mole of oxygen uptake were precipitated at pH 5.2. The precipitate was collected in the centrifuge and the sediment was washed first with water (adjusted to pH 5.2), then with alcohol, and

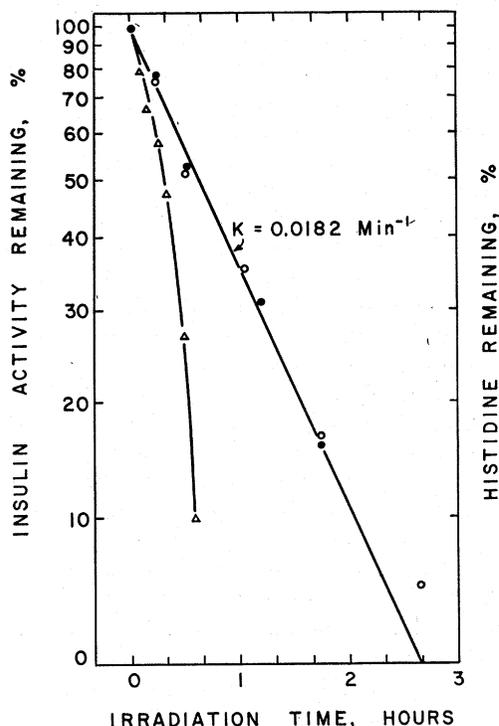


FIG. 8. Loss of histidine and biological activity of insulin as function of length of irradiation. ○—○, Histidine; ●—● biological activity; △—△, free histidine. The loss of histidine due to photooxidation was estimated by amino acid analysis.

finally with ether. The powder was dried *in vacuo*. The disulfide bonds were cleaved with performic acid (11). The B-chain, containing the histidine residues, was isolated by the procedure of Mycek *et al.* (10). The yield of B-chain from native insulin was 55%, while from photooxidized insulin it was 40%. Sixty-mg quantities of B-chain derived from the native and photooxidized insulin were subjected to the action of 0.6 mg of crystalline pepsin at pH 2.0 (total volume 12 ml) for 24 hours at 37°. The reaction was stopped by lyophilization. The separation of the histidine-containing peptides was accomplished by high voltage paper electrophoresis by means of Ingram's procedure (12). Aliquots containing 5 mg of hydrolyzate were placed on Whatman 3 MM paper soaked with pyridine-acetate buffer of pH 6.4 and subjected to electrophoresis at 3000 V for 2.5 hours. After the

paper was dried, the bands were stained with ninhydrin and marked with pencil. The ninhydrin color was then bleached by dipping the strip in 1 N HCl-acetone solution (1:3) and allowing it to dry. The dry strip was cut in half lengthwise. One-half was treated to locate tyrosine peptides with α -nitro- β -naphthol and HNO_3 [which is specific for tyrosine (13)], and the second half for histidine peptides (13) with sulfanilic acid and HNO_2 . Diazotized sulfanilic acid gives a bright orange color with histidine peptides and in some cases also produces a positive reaction with tyrosine peptides, but of a distinctly different shade. The high voltage electrophoresis patterns obtained at pH 6.4 from the B-chains of native and photooxidized insulin (1 mole of oxygen per mole of protein) were identical (Fig. 9). Bands 1, 2, 4, and 5 gave positive reactions for tyrosine. Only band 3 gave a distinct bright orange color with diazotized sulfanilic acid characteristic of histidine peptides; and although band 4 gave a positive reaction for histidine, it was faint and distinctly of a different shade. Since only band 3 gave positive histidine and negative tyrosine reactions, preparative electrophoretic runs were repeated under the above conditions with 10-mg aliquots. After the paper was dried, a guide strip was cut off the edge and the bands were located by ninhydrin. The sections corresponding to band 3 were cut out and the peptide was eluted with 1 M acetic acid. The eluates were combined and evaporated to dryness in vacuum. The peptides isolated in this manner migrated as one spot whether the high voltage electrophoresis took place at pH 6.4 or 1.9.

Amino acid analysis of the histidine peptides isolated from native and partially

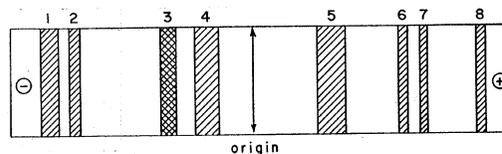


FIG. 9. High voltage electrophoresis diagram of peptic hydrolyzate of B-chain derived from native and photooxidized (1 mole O_2 per mole of insulin) insulin at pH 6.4 (unbuffered).

photooxidized insulin showed the compositions:

Native: Asp_{1.03} Ser_{0.87} Glu_{0.98} Gly_{1.0} CysSO₃
 H_{1.05} Val_{1.03} Leu_{2.02} Phe_{0.92} His_{1.96}
 Photooxidized: Asp_{1.1} Ser_{1.1} Glu_{1.0} Gly_{1.1}
 CysSO₃ H_{0.95} Val_{1.03} Leu_{1.96} Phe_{0.82} His_{1.94}

It is clear that peptides containing photooxidized histidine are not detected by diazotized sulfanilic acid. On the basis of the known covalent structure of insulin (1-3) the sequence of the peptide is:

NH₂-Phe.Val.Asp NH₂.Glu NH₂.His.Leu.
 Cys.Gly.Ser.His.Leu.COOH

The fact that only unmodified peptide was isolated from partially photooxidized insulin supports the results of Fig. 8, which suggest that the two histidine residues in insulin are photooxidized simultaneously.

Effect of photooxidation on the solvent perturbation difference spectra of Zn-free insulin and Zn-insulin. The effect of photooxidation on the solvent perturbation difference spectra as well as the optical rotatory

properties of insulin were examined to ascertain that the observed inactivation was not due to changes in the secondary and tertiary structure of the hormone.

Photooxidation of insulin and the removal of dye were carried out as described for experiments presented in Fig. 1. The solvent perturbation technique employed in obtaining difference spectra has been described by Herskovits and Laskowski (14, 15). The measurements were carried out in 1-cm "tandem" cells designed to correct for the contribution of the solvent and perturbant to the difference spectra. Twenty % sucrose was employed as a perturbant in these experiments. Protein concentrations were based on total nitrogen analysis (15.87 % N being the calculated value) and on absorbancy measurements. A graphical presentation of the results obtained is shown in Fig. 10. Table II summarizes the numerical values of the difference spectral parameters of the photooxidized insulin. The parameter R_M is a measure of the fraction of exposed tyrosine residues to the perturbant, sucrose

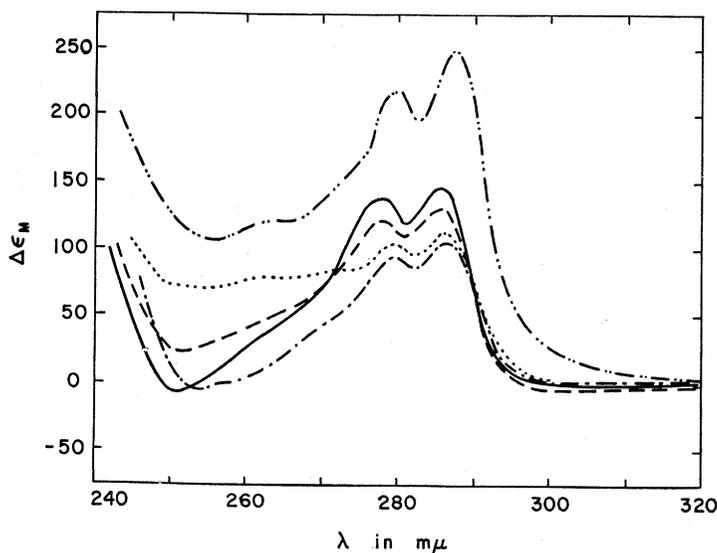


FIG. 10. The effect of photooxidation on the solvent perturbation difference spectra of zinc and zinc-free insulin. —, Zn-free insulin in 0.025 *M* phosphate, pH 7.0; ---, Zn-free insulin + 2 moles of O₂ in 0.025 *M* phosphate, pH 7.0; - · - ·, Zn-insulin in 0.025 *M* phosphate, pH 7.8; · · ·, Zn-insulin + 2 moles of O₂ in 0.025 *M* phosphate, pH 7.4; - - - - -, Zn-free insulin in 8 *M* urea, 0.05 *M* Cl⁻, 0.04 *M* thioglycolic acid, pH 3.4. Protein concentration, 0.22-0.25%.

TABLE II
EFFECT OF PHOTOOXIDATION ON DIFFERENCE
SPECTRAL PARAMETERS OF INSULIN

Protein	Extent of Photooxidation ^a	pH ^b	$\Delta\epsilon_M^c$ (286-38m μ)	R_p^d
Zn-free insulin	0	7.0	160 \pm 10	0.67 \pm 0.07
do.	1	7.0	150	0.63
do.	2	7.0	150	0.63
Zn-insulin	0	7.8	110	0.46
do.	1	7.4	120	0.50
do.	2	7.4	120	0.50
Disulfide cleaved insulin in 8 M urea ^e	0	3.4	240	1.00

^a Moles of oxygen per mole of insulin.

^b pH 7.0-7.8, 0.025 M phosphate.

^c Molar difference absorption coefficient, obtained with 20% sucrose as perturbant.

^d Relative $\Delta\epsilon_M$, taking the disulfide cleaved unfolded protein in 8 M urea as a reference to estimate the fraction of exposed tyrosyl residues (14, 15).

^e Thioglycolic acid reduced insulin in 8 M urea, 0.05 M Cl⁻, 0.04 M thioglycolate.

(14, 15). It is defined as the $\Delta\epsilon_M$ of insulin relative to the $\Delta\epsilon_M$ value of the hormone in the disulfide-cleaved fully unfolded state, in 8 M urea, where all the tyrosine residues are exposed to solvent.

Effect of photooxidation on the optical rotatory parameters of Zn-free insulin. Optical rotatory dispersion measurements were made in a Rudolph model 200S spectropolarimeter. The rotatory dispersion data were interpreted by means of the Moffitt-Yang equation (16, 17):

$$[m'] = 3 M_0/100(n^2 + 2)[\alpha]_\lambda \\ = a_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + b_0\lambda_0^4/(\lambda^2 + \lambda_0^2)^2$$

The parameters a_0 and b_0 were calculated from the intercepts and slopes of $[m'](\lambda^2 - \lambda_0^2)$ versus $(\lambda^2 - \lambda_0^2)^{-1}$ plots, with λ_0 taken as 212 m μ and M_0 , the mean residue rotation, taken as 112. Conditions for the photooxidation and removal of the dye were the same as those described for experiments presented in Fig. 1. It should be noted that the removal of the last traces of dye requires particular attention, since this very small amount of residual dye tends to lower the calculated b_0 values. Figure 11 shows the graphical presentation of the results ob-

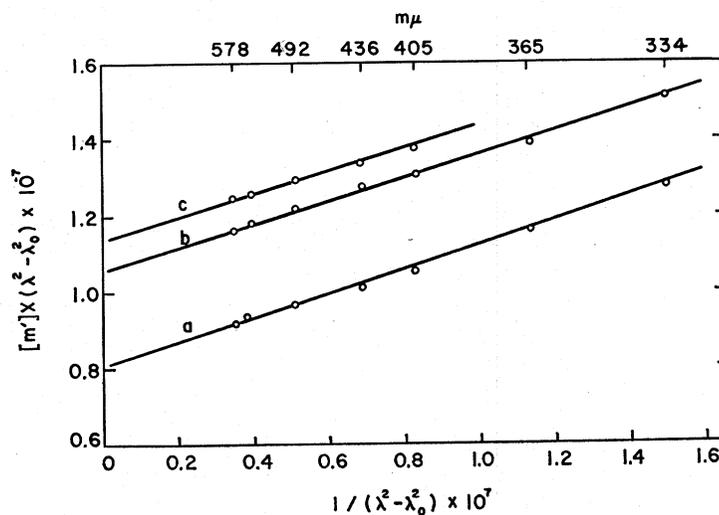


FIG. 11. Moffitt-Yang plots of native and photooxidized Zn-free insulin. *a*, Zn-free insulin; *b*, Zn-free insulin photooxidized to the extent of 1 mole of oxygen per mole of protein; *c*, Zn-free insulin photooxidized to the extent of 2 moles of oxygen per mole of protein. Buffer, 0.02 M phosphate, pH 7.0. Protein concentration, 0.41-0.52%.

TABLE III
EFFECT OF PHOTOOXIDATION ON THE OPTICAL
ROTATORY PARAMETERS OF INSULIN

	Extent of Photo- oxida- tion ^a	pH ^b	a_0	b_0
Zn-free insulin	0	7.0	-180 ± 10	-160 ± 10
do.	1	7.0	-235	-150
do.	2	7.0	-255	-145
Zn-insulin	0	7.8	-180	-200

^a Moles of oxygen per mole of insulin.

^b pH 7.0, 0.025 M phosphate; pH 7.8, 0.1 M Cl⁻.

tained, and Table III shows the optical rotatory parameters of the photooxidized insulin.

DISCUSSION

Studies to correlate structure with insulin activity have been reported in the literature. Thus, Harris and Li (18) and Nicol (19) have noted that removal of the C-terminal alanine from the B-chain of insulin by carboxypeptidase A could be accomplished without diminution in biological activity. On the other hand, cleavage of the C-terminal asparagine of the A-chain resulted in a complete inactivation (18, 19). Sulfation of the hydroxy group of serine (20), esterification of two thirds of the carboxyl groups (21), and modification of the amino groups (22) of insulin do not appear to affect its physiological activity. Similarly, conversion of the single lysine residue in the B-chain of insulin to homoarginine (23) and hydrolysis of the first six amino acid residues from the N-terminus of the B-chain of insulin by aminopeptidase, according to Smith *et al.* (24), had no influence on its hypoglycemic action. On the other hand, hydrolysis of insulin by trypsin, which results in the release of alanine due to the splitting of the 29-30 bond in the B-chain and formation of heptapeptide released by a further split at bond 22-23 in the B-chain, afforded a "deso-octa-peptide-insulin" completely devoid of biological activity (25, 26). Separation of the A- and B-chain of insulin after sulfite cleavage and subsequent reduction of the individual chains to the sulphydryl form

produced inactive preparations (27), but the mixture of the two chains upon oxidation produced statistically significant regeneration of biological activity (27).

Although studies on modifications of the insulin molecule in which biological activity is retained are useful in the sense that they point out certain unessential features of the hormone, in those cases where such modification results in elimination of biological activity, the interpretation is not as simple. It then becomes essential to establish that modifications produced in the molecule are directly related to biological activity and do not exert their effects because of over-all changes in the three-dimensional structure of the molecule.

In view of these considerations, experiments were undertaken to study the effect of photooxidation of bovine insulin sensitized by methylene blue. Photooxidation studies on bovine insulin are particularly useful since the hormone contains only two photo-reactive amino acids, namely, histidine and tyrosine, while tryptophan and methionine are absent. Previous studies (7) have shown that the photooxidation of tyrosine can be repressed by lowering the temperature and pH. Thus, by carrying out the photooxidation of insulin at 10° and at pH 7.0, changes in the amino acid composition of insulin are confined solely to the two histidine residues (see Table I). These changes are also fully reflected in the ultraviolet absorption spectra (see Fig. 1). While the reaction at 2° and 10° is confined to the two histidine residues, at 20° and 40° there is additional participation of tyrosine residues in the photooxidation process (Fig. 2).

The importance of the conformation of insulin to photooxidation becomes apparent when the rate and extent of oxidation are compared in water and 8 M urea solution. Although the extent of photooxidation is limited to about 2 moles of oxygen per mole of insulin in the water solution, in the 8 M urea solution both the rate and extent of photooxidation increase (Fig. 3A), which is indicative of an exposure of both histidine and tyrosine residues. In experiments carried out simultaneously with a model amino acid mixture (histidine and tyrosine mixture, molar ratios = 2:4) the same reaction rate

as with insulin in urea solution is observed up to 2 moles of oxygen uptake where, presumably, only the two histidines have reacted, but above this level of oxygen uptake the reaction rate of the model amino acid mixture is above that observed for insulin in urea solution. It would appear that the two histidine residues of insulin in urea solution become fully exposed and are as photo-reactive as the free amino acid, while the additional slower photooxidation of tyrosine residues might be indicative of a retention of a certain degree of structure of the protein in 8 *M* urea, or that the tyrosine side-chains, even if fully exposed, are oxidized at a somewhat slower rate. In similar experiments a stoichiometric amount of A-chain, which contains two tyrosine residues, as anticipated reacted very sluggishly in water, and only a very slight increase in oxidation rate could be observed in 8 *M* urea solution (Fig. 3B). However, on photooxidation the corresponding model amino acid solution (i.e., a stoichiometric amount of tyrosine) reacted at a considerably faster rate. It would appear that the two tyrosine residues in the A-chain are not as fully exposed as in native insulin. On photooxidation, the B-chain (Fig. 3C), which contains two histidine and two tyrosine residues, reacted in aqueous solution at a faster rate than insulin (Fig. 3A). This is indicative of a loosening of the original structure which became more pronounced in 8 *M* urea solution. Figure 3D, which represents the sum of the curves of Fig. 3B and 3C, is in fairly good agreement with the individual observations (Fig. 3A-3C) inasmuch as the sum of the rates of the model amino acid mixture for the A- and B-chains is in close agreement with that obtained with the model amino acid mixture for insulin (Fig. 3A). However, the sum of the rates for the A- and B-chains in 8 *M* urea (Fig. 3D) is below that obtained with insulin in the same solvent (Fig. 3A), and indicates, for reasons not yet clear, that the tyrosine residues in the individual chains are less accessible to photooxidation than in insulin itself.

The observed correlation between the destruction of biological activity and the photooxidation of two histidine residues in the B-chain (Fig. 7) implicates this amino

acid in the biological action. Both phenomena follow identical first-order kinetics (Fig. 8). The results suggest that both histidine residues are photooxidized simultaneously. This view is also supported by the fact that only one peptide-containing histidine can be isolated from peptic hydrolyzates of the B-chains (Fig. 9) from native and partially photooxidized insulin (i.e., NH₂-Phe.Val.Asp - NH₂.Glu - NH₂.His.Leu.Cys. Gly.Ser.His.Leu.COOH). The question whether only one or both histidine residues are related to the hormonal function remains to be answered. Smith *et al.* (24) showed that the first six amino acid residues (with histidine in position 5) from the N-terminus of the B-chain could be removed enzymically without impairment of biological activity. Thus it is possible that the loss in biological activity is related to the photooxidation of only one histidine, that in position 10 of the B-chain.

The possibility that photooxidation may produce changes in the secondary and tertiary structure which by themselves could account for the observed loss in biological activity has been considered. We have studied the rotatory dispersion and the solvent perturbation difference spectral properties of insulin prior to and following photooxidation. Rotatory dispersion measurements may be interpreted in terms of possible changes in the secondary structure (helix content) of insulin, while the difference spectral technique (14, 15) leads to information about possible changes in the environment of chromophoric side-chains; that is, tyrosyl residues in the case of insulin (Table II and Fig. 10). From the difference spectral data it is concluded that the fraction of tyrosyl residues buried in the interior folds of insulin remains essentially the same after photooxidation. In Zn-insulin about 50%, or two of the four tyrosyl residues, seem to be buried, as is suggested by the $\Delta\epsilon_M$ values of native insulin compared to the $\Delta\epsilon_M$ value of fully unfolded, disulfide-cleaved protein in 8 *M* urea solution. It is noteworthy that removal of Zn from insulin results in the exposure of nearly one additional tyrosyl group (Table II). This change in the conformation of Zn-free insulin is also reflected by a less negative Moffitt b_0 parameter

[about -150 for Zn-free and -190 to -200 for Zn-insulin, depending on pH (this work and Ref. 28)]. Both of these findings suggest that Zn-free insulin is somewhat more unfolded and is less helical than is Zn-insulin. Moreover, this conclusion is in accord with the observation that the rate of photooxidation of Zn-free insulin is considerably faster than that of Zn-insulin (Fig. 5).

The results of the rotatory dispersion measurements (Table III and Fig. 11) support the conclusion obtained by difference spectral studies that the photooxidation of insulin is not associated with changes in the secondary and tertiary structure of the protein, and indicate that the observed proportionality between decrease of histidine and loss of biological activity during photooxidation can be related to this amino acid.

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