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Photoöxidation of Crystalline β-Lactoglobulin in the Presence of Methylene Blue

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In previous studies (1,2), photoöxidation of the various amino acids mediated by visible light in the presence of methylene blue was investigated. We have extended our work now to the study of the amino acid residues within the framework of the protein molecule, and this paper reports our findings on crystalline β-lactoglobulin.

Experimental

Throughout the experiments, three-times crystallized β-lactoglobulin, prepared according to the method of Palmer (3), was used. Irradiation by visible light and measurement of oxygen uptake and CO_2 evolution were carried out as described previously (4).

Influence of pH on the Photochemical Action of Methylene Blue on β-Lactoglobulin

In all the experiments, the β-lactoglobulin concentration was calculated from the total nitrogen concentration, based on the nitrogen content of β-lactoglobulin as 15.60% (5). The protein dissolved in 0.1 *N* NaCl was adjusted to pH values of 4.5, 5.8, 6.8, 7.8, 8.7, and 9.2, respectively, with 0.1 *N* HCl or 0.1 *N* NaOH. The final solutions contained 10 mg. protein/ml. To 1 ml. of the solution was added 0.5 ml. of an 0.1 *N* NaCl solution containing 0.1 mg. of methylene blue. The latter was placed in the side arm of the Warburg vessel, and after temperature equilibrium at 37° it was tipped into the main chamber, and the irradiation was started. Because of the CO_2 evolution during the photoöxidation as shown later, KOH was placed in the center well of the Warburg vessel in all experiments unless otherwise stated. Results presented in Fig. 1 show that the optimum pH for the photoöxidation of β-lactoglobulin is about 8.7, a value similar to that observed for the photoöxidation of histidine, tryptophan, tyrosine, methionine, and cystine (2). At pH 4.5 and 5.8, near the isoelectric point of the protein, formation of a precipitate was observed.

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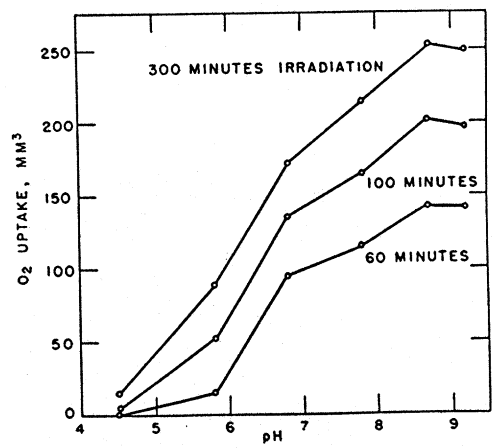

FIG. 1. Influence of pH on the photochemical action of methylene blue on β-lactoglobulin.

Effect of Various Amounts of Methylene Blue on the Photochemical Action of β-Lactoglobulin

To 10 mg. β-lactoglobulin dissolved in 1 ml. of 0.1 *N* NaCl (adjusted to pH 8.5), 0.5 ml. of 0.2 *M* phosphate buffer (pH 8.5) and 0.5 ml of 0.1 *N* NaCl solution containing 0.025–0.5 mg. methylene blue were added, and the mixtures were irradiated as described before. The results n Fig. 2 show that the optimal dye concentration lies between 0.05

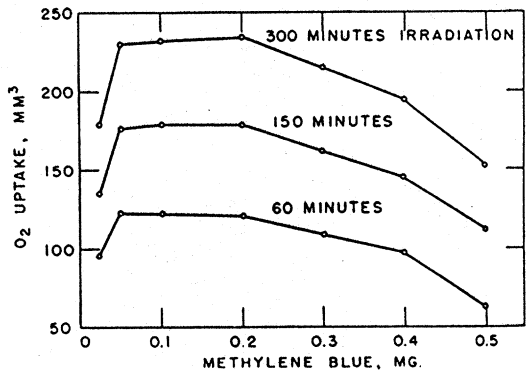

FIG. 2. Influence of different amounts of methylene blue on the photoöxidation of β-lactoglobulin.

and 0.2 mg. Below and above these concentrations a decline in the oxygen uptake was observed. The possible interpretation of this observation was advanced in our previous paper (4).

Photochemical Action of Methylene Blue on β-Lactoglobulin in Air, Oxygen, and at Various Temperatures

The composition of the reaction mixture was the same as that described for the experiments presented in Fig. 2, except that the dye concentration was 0.1 mg. One Warburg vessel was flushed with oxy-

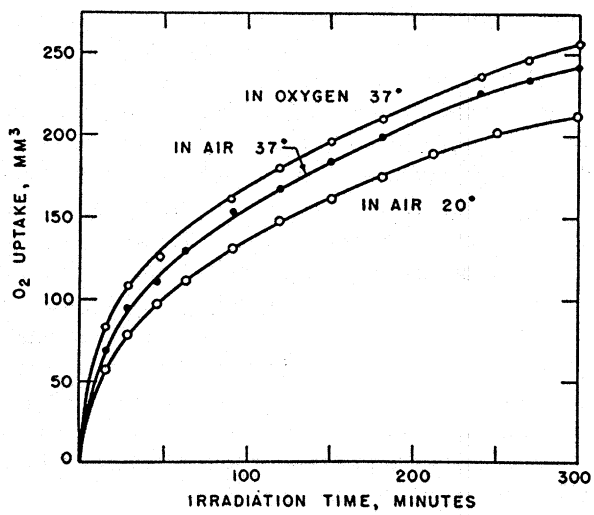

FIG. 3. Influence of oxygen concentration and temperature on the photochemical action of methylene blue on β-lactoglobulin.

gen; in the second, air was used. The results in Fig. 3 show only a slight acceleration in oxygen uptake when pure oxygen was used, in comparison with the results when irradiation was carried out in air. Because of this slight difference, all the succeeding experiments were carried out in air. Figure 3 includes a duplicate experiment carried out in air at 20° instead of 37°. The relatively small difference in the reaction rates of these two temperatures indicates that the rate-controlling step in the photoöxidation of β-lactoglobulin is photochemical in nature.

Oxygen Uptake, CO_2 Evolution, and the Formation of an Intermediary Oxidizing Agent During the Photochemical Action of Methylene Blue on β-Lactoglobulin

The procedure followed was as described for the experiments presented in Fig. 3 except that 11.7 mg. of β-lactoglobulin was irradiated. The CO_2 evolution was determined by the two-vessel method, one having concentrated KOH in the center well. After 360 min. irradiation, the reaction practically came to a standstill; the oxygen uptake then amounted to 293 cu. mm., and the measured CO_2 evolution was 80 cu. mm., resulting in an R.Q. of 0.27. The result indicates that during the photoöxidation of β-lactoglobulin the oxygen uptake is associated with CO_2 evolution. In the light of our previous work (2), this CO_2 evolution is chiefly due to the photoöxidation of the aromatic nucleus of tryptophan, tyrosine, and partly, cystine. A similar CO_2 evolution was observed by Gaffron (6) during the photoöxidation of casein and serum. In our previous work (2,4), the possible intermediary formation of H_2O_2 or organic peroxide could be demonstrated by adding Na pyruvate to the irradiation mixture. The formed intermediate reacted with the latter, forming Na acetate and CO_2. The CO_2 evolution in the presence of Na pyruvate was considered indicative of peroxide formation. To support this point, experiments were carried out similar to the ones described for the demonstration of CO_2 formation only in the presence of 1 mg. of Na pyruvate. The observed oxygen uptake under this condition was 287 cu. mm., essentially the same as that without Na pyruvate, but the CO_2 evolution was doubled (164 cu. mm.) and resulted in an R.Q. of 0.54, indicating formation of an intermediary oxidizing agent. A control experiment with Na pyruvate and the dye showed no oxygen uptake or CO_2 evolution.

Photochemical Action of Methylene Blue on the Histidine, Tryptophan, Tyrosine, Cystine, and Methionine Content of β-Lactoglobulin

In previous studies, we have shown that histidine, tryptophan, tyrosine, cystine, and methionine are highly susceptible to the photochemical action of methylene blue (1,2). In view of these findings, the fate of these five amino acids was investigated during the various degrees of photoöxidation of β-lactoglobulin.

Histidine determinations were carried out by the microbiological method of Stokes *et al.* (7); *Streptococcus faecalis* was used as the organ-

ism. Methionine was determined by the method of McCarthy and Sullivan (8). Preliminary studies showed that when histidine was fully photoöxidized, that is, if 1 mole of amino acid took up 1 mole of oxygen, it did not respond to the microbiological assay designed for its determination, and thus the above-mentioned method could be used for the measurement of its photochemical decomposition. For our irradiation studies, 10 mg. β-lactoglobulin was used as described for the experiments presented in Fig. 3. At various stages of oxygen uptake, the contents of the Warburg vessel were transferred to a small vial, to which concentrated HCl was added to make the acid concentration 10%, and after the tube was sealed, the reaction mixture was hydrolyzed for 10 hr. at 15 lb. pressure. The hydrolyzate then was transferred and adjusted to pH 6.8, and the dye was removed with a small amount of charcoal. The resulting filtrate was diluted as desired and assayed according to the method mentioned (7). Slight losses due to the charcoal treatment were determined by total nitrogen determination.

Tryptophan was determined by the method of Shaw and McFarlane (9) on the unhydrolyzed protein. For the estimation of tyrosine, Bernhart's (10) modification of the Millon-Weiss reaction was used. Cystine (which included both the —SH and S—S forms) was determined by the Buechler and Lollar (11) modification of the Sullivan, Hess, and Howard method (12). The oxygen uptake per mole of β-lactoglobulin was calculated on the basis of a molecular weight of 42,020, the value of Brand *et al.* (5). Although other molecular weights reported in the literature (13,14,15) differ from this value, they do not alter the general interpretation of our result. The results (Fig. 4) show that histidine is the most susceptible amino acid to photoöxidation and was completely photoöxidized when 1 mole protein took up about 10 moles oxygen. At this point, about 50% of the total tryptophan was gone. The complete elimination of tryptophan took place after about 20 moles oxygen was taken up by 1 mole protein. Tyrosine, as can be seen from Fig. 4, remained unchanged until all the histidine and tryptophan were photoöxidized, after which point a gradual decrease took place, reaching a decrease of about 62% at 48 moles oxygen uptake/mole protein. Further irradiation beyond that point produced practically no additional oxygen uptake. The decrease of cystine during the irradiation was progressive but slow, amounting to about 15% at 20 moles oxygen uptake, whereas at 48 moles oxygen uptake it was about 37%. Methionine, which in our model experiments (1,2) was photoöxidized rapidly,

surprisingly was not affected at all during the irradiation. It would appear that the long peptide chain in which methionine is incorporated exerts a protective influence because of some unknown spatial arrangement.

Effect of the Photochemical Action of Methylene Blue on the Ultraviolet Absorption Spectrum of β-Lactoglobulin

Ten mg. β-lactoglobulin was irradiated as described for the experiments presented in Fig. 3, and the ultraviolet absorption spectrum was measured at various levels of oxygen uptake. The dye was removed by a small amount of charcoal, and the resulting filtrate was analyzed for total nitrogen, to correct for the slight losses due to adsorption. The

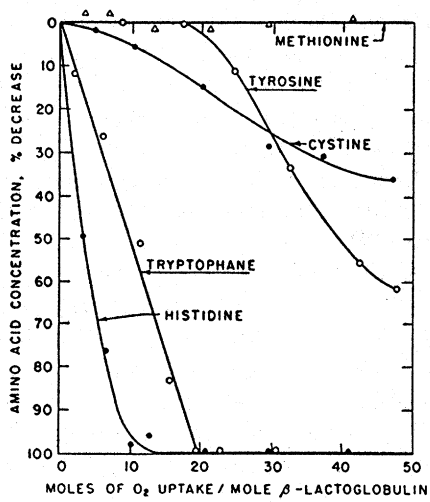

FIG. 4. Decrease of various amino acids during the photochemical action of methylene blue on β-lactoglobulin.

results obtained (Fig. 5) show the characteristic absorption maximum of 279 mμ and a minimum at 250 mμ for the original β-lactoglobulin. At 8 moles oxygen uptake/mole protein, the maximum, with a somewhat lower extinction coefficient, shifted to 272 mμ, and the minimum, with a higher extinction coefficient, to 254 mμ. At 18 moles oxygen uptake (Fig. 4), practically all histidine and tryptophan were gone; the maximum shifted further to 264 mμ, and the minimum shifted to 255

mμ. Simultaneously with these shifts, a general increase in absorption at all wavelengths was noted. With further oxygen uptake, the absorption minimum became stationary, but a slow shift of the maximum toward the shorter wavelengths took place, reaching 260 mμ at 30 moles oxygen uptake/mole protein. The progressive increase in absorption at all wavelengths was also observed. At 39 moles oxygen uptake no absorption maximum or minimum could be observed. The same experiment carried out in nitrogen and irradiated for 360 min. showed

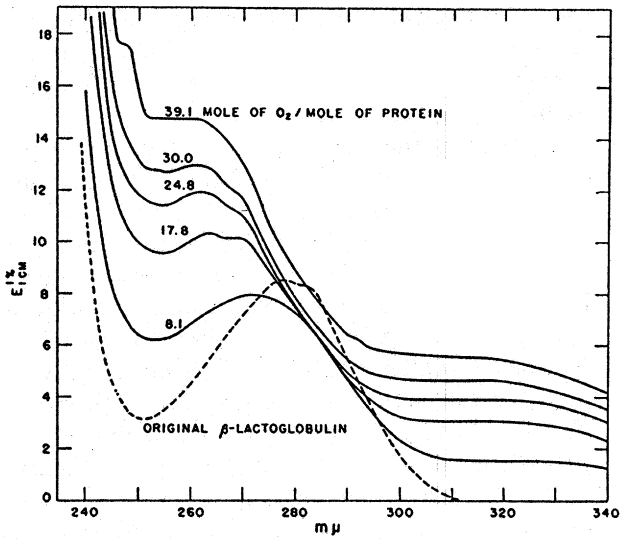

FIG. 5. Changes in the ultraviolet absorption spectrum of β-lactoglobulin produced by the photochemical action of methylene blue.

no difference in the ultraviolet absorption spectrum from that of the original β-lactoblobulin, although a distinct bleaching of the dye could be observed.

Photochemical Action of Methylene Blue on the Solubility and Viscosity of β-Lactoglobulin

To study the changes in solubility of β-lactoglobulin produced by the photochemical action of methylene blue, irradiation was carried out at the isoelectric point (pH 5.2) of the protein. To 12 mg. of the protein dissolved in 1.5 ml. of 0.1 *N* NaCl was added 0.1 mg. methylene

blue dissolved in 0.5 ml. of 0.1 N NaCl, and the solution was irradiated as described before.

During the irradiation, with progressive oxygen uptake a corresponding increase of precipitate formation was observed. The irradiation time required for 25 moles oxygen uptake/mole protein was 240 min. At given levels of oxygen uptake, aliquots were removed, and after being cooled in ice for 1 hr. were filtered through sintered glass. The clear filtrate was analyzed for total nitrogen. The results in Fig. 6 show that progressive oxidation produced a decrease in solubility of the protein, which reached complete insolubility at about 19 moles oxygen uptake/mole protein. The decrease in solubility coincided approximately with the decrease of the tryptophan content of β-lactoglobulin (Fig. 4).

Essentially the same result was obtained where the photoöxidation was carried out at pH 8.5 and the pH was adjusted after photoöxidation to 5.2. In the latter case, however, a correction factor had to be applied owing to the partial solubility of the irradiated protein, caused by the dilution required in this procedure. Since the decrease in solubility of β-lactoglobulin during photoöxidation indicates some kind of aggregation of the molecule, the photochemical action of methylene blue on the protein was followed viscometrically. Because of the depressing action of anions on viscosity (16), phosphate buffer was omitted. To 27.9 mg. of β-lactoglobulin dissolved in 0.1 N NaCl and adjusted to pH 7.3 (total volume 1.5 ml.), 0.1 mg. of the dye dissolved in 0.5 ml. of 0.1 N NaCl was added, and the solution was irradiated. At various degrees of oxygen uptake, 1 ml. of this reaction mixture was transferred to an Ostwald type viscometer with a capacity of about 0.5 ml. and a flow time for 0.1 N NaCl solution of 107 sec. at 25°. The relative viscosities (Fig. 6) are in agreement with the solubility measurements inasmuch as increased oxygen uptake increased the relative viscosity, with a simultaneous decrease of solubility. In both cases, no further changes could be observed after 19–20 moles oxygen uptake/mole protein.

Identical experiments were carried out on pH-denatured β-lactoglobulin. To the crystalline protein dissolved in 0.1 N NaCl, borate buffer of pH 8.7 was added until the ionic strength was 0.1; the solution was kept in the refrigerator for 4 days. The pH then was adjusted to 5.2, and the denatured protein was centrifuged. The resulting sediment was washed twice by resuspending it in 0.1 N NaCl solution and centri-

fuging. The final sediment was resuspended in 0.1 N NaCl solution and brought into solution by adjusting the pH to 7.3. The experimental conditions for the irradiation and viscometric measurement of the pH-denatured β-lactoglobulin were the same as those described for the native protein. Results in Fig. 6 show that the pH-denaturation of β-lactoglobulin prior to irradiation did not eliminate the increase in viscosity due to irradiation but instead produced an additional increase. In contrast to the results obtained with the native protein, the observed increase in viscosity did not stop at 20 moles oxygen uptake/mole protein. Control experiments carried out on the protein samples without the addition of methylene blue, but otherwise irradiated the same

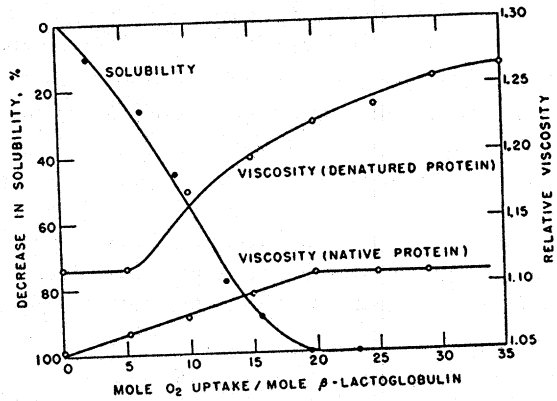

FIG. 6. Changes in solubility and viscosity of β-lactoglobulin produced by the photochemical action of methylene blue.

way and for the same length of time, did not show any decrease in solubility or increase in viscosity.

Molecular weight determinations made by Dr. M. Halwer of this laboratory by the light-scattering method on a β-lactoglobulin sample which had been photoöxidized to the extent of 20 moles oxygen/mole protein gave an average value of 147,000, indicating a considerable increase in molecular weight.

Photochemical Action of Methylene Blue on Native and Heat-Denatured β-Lactoglobulin

β-Lactoglobulin was denatured by dissolving it in 0.1 N NaCl solution and heating it in boiling water for 10 min. The precipitate was

centrifuged and washed with 0.1 N NaCl solution; the sediment was resuspended again in 0.1 N NaCl solution and adjusted to pH 8.5 by addition of 0.1 N NaOH. The undissolved particles were centrifuged, and the supernatant clear protein solution was used. As described for the experiment presented in Fig. 3, 5.6 mg. of the native and heat-denatured β-lactoglobulin was used, and each was irradiated at pH 8.5. The result showed that the oxygen uptake for both proteins proceeded exactly at the same rate, indicating that the heat denaturation prior to photoöxidation had no influence on the oxygen uptake of β-lactoglobulin.

Is the Peptide Bond Hydrolyzed During the Photochemical Action of Methylene Blue on β-Lactoglobulin?

For this purpose, 15 mg. β-lactoglobulin was irradiated as described for the experiments presented in Fig. 3, to the extent of 10, 20, 30, and 40 moles oxygen uptake/mole protein. The possible increase of α-amino nitrogen due to hydrolysis was measured by the manometric Van Slyke procedure. In no instance could we observe any increase in the α-amino nitrogen, indicating that no hydrolysis of the peptide bonds caused by photoöxidation took place. Similar findings were observed recently by McLean and Giesse (17), who used ultraviolet irradiation.

Is Any Nitrogenous Substance Released During the Photochemical Action of Methylene Blue on β-Lactoglobulin?

Experiments on the photoöxidation were carried out in the same way as the preceding experiments. After various degrees of oxygen uptake, 2 ml. of a 20% solution of trichloroacetic acid was added to the Warburg vessel, and the mixture was then transferred and centrifuged. The supernatant fluid was analyzed for nitrogen. The results, presented in Table I, show that during the photoöxidation of β-lactoglobulin, there was a slight increase of nitrogenous substances which could not be precipitated by trichloroacetic acid.

TABLE I

Formation of Non-Protein Nitrogen During the Photochemical Action of Methylene Blue on β-Lactoglobulin

Moles of O_2 taken up per mole of protein	0	13.2	25.8	37.2	44.0
Per cent nitrogen in solution	0.0	1.16	1.24	1.56	1.58

Photochemical Action of Methylene Blue on Lysozyme, Insulin, Salmine, and Gelatin

Ten mg. of crystalline lysozyme (Armour Company),[2] crystalline insulin (Eli Lilly Company), salmine sulfate, and gelatin (Difco) were photoöxidized as described for the experiments presented in Fig. 3. The concentrations of these proteins were calculated on the basis of the total nitrogen concentration of 18.6% for lysozyme (18); 16.04% for insulin (19); 24.8% for salmine (20); and 17.6% for gelatin (21). The results, presented in Fig. 7 together with similar data for β-lactoglobulin for comparison, show that lysozyme took up the greatest amount of

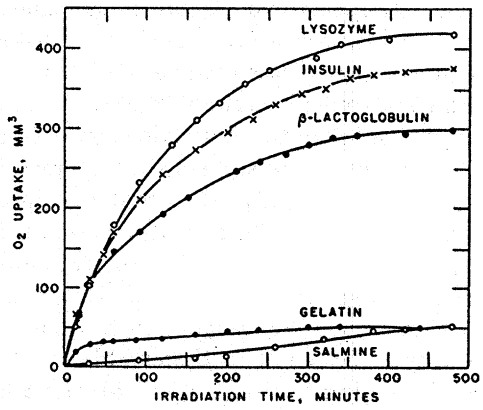

FIG. 7. Photochemical action of methylene blue on lysozyme, insulin, β-lactoglobulin, gelatin, and salmine.

oxygen per unit weight, followed by insulin and β-lactoglobulin. The qualitative relationship between the oxygen uptake of these proteins and the concentration of the amino acids which were found to be photochemically reactive (2) is apparent. A quantitative relationship, however, would require a more detailed investigation.

Salmine, which does not contain any of the photochemically reactive amino acids (histidine, tryptophan, tyrosine, methionine, and cystine), and gelatin, which has a low content of these amino acids, took up only small amounts of oxygen.

[2] Mention of products and companies in this paper does not constitute an endorsement by the Department of Agriculture over others not mentioned.

Discussion

The photochemical action of various dyes on the amino acid residues within the protein molecule has been investigated by various workers (6, 22–26), and there appears to be general agreement that tryptophan, tyrosine, and histidine units of the protein molecule are particularly susceptible in this reaction. These observations were confirmed by our investigation and were further extended in order to establish a quantitative relationship between the oxygen uptake of the protein and the changes in amino acid concentration during the photochemical action of methylene blue on β-lactoglobulin.

Our results indicate that the first point of attack of the photochemical action of methylene blue on β-lactoglobulin takes place on the histidine and tryptophan residues; the former are photoöxidized at a more rapid rate, reaching zero concentration at about 10 moles oxygen uptake/mole protein. At about 20 moles oxygen uptake, tryptophan, too, disappears. No decrease in tyrosine concentration is observed up to this point, and a gradual decrease takes place only after the oxygen uptake/mole protein passes the 20-mole mark. It appears that histidine and tryptophan exert a protective action on tyrosine, the latter becoming susceptible to photoöxidation only after the first two amino acids are eliminated. In spite of the high reactivity of cystine (which includes both the —SH and S—S forms) in our model experiments (2), it is photoöxidized slowly. The slow decrease of this amino acid may be due to its spatial arrangement within the framework of the protein molecule. A more pronounced example of this phenomenon is seen in methionine, which in the β-lactoglobulin molecule is not affected by the photochemical action of methylene blue.

According to Brand *et al.* (5), the β-lactoglobulin molecule contains 4 tryptophan,[3] 4 histidine, 9 tyrosine, and 6 cystine residues. Our previous studies (2) have shown that the photoöxidation of these amino acids requires 4, 1, 2, and 3.5 moles oxygen, respectively, per mole of amino acid. On this basis, one might study the quantitative relationship between the oxygen uptake and decrease of these amino acids during the photoöxidation of β-lactoglobulin. We have shown that at 20 moles oxygen uptake/mole protein, histidine and tryptophan are completely photoöxidized, whereas at this point tyrosine is not affected and only a 15% decrease of cystine is observed. The calculated moles of oxygen required for the photoöxidation of these amino acids would

[3] Recent work of Spies and Chambers (27) reported a higher value.

be 23, which is in fairly good agreement with our experimental value of 20 moles. At 48 moles oxygen uptake/mole β-lactoglobulin, the calculated oxygen value, based on the observed decrease of amino acids, would be 38 moles. The higher observed value might be due to the slow reactivity of the other amino acids (2) when the irradiation was carried out over a longer period.

The photoöxidation of β-lactoglobulin was associated with a progressive aggregation of the molecule, as indicated by the solubility and viscometric studies. A progressive decrease in solubility and a progressive increase in viscosity were observed during the photochemical action of methylene blue on β-lactoglobulin up to the point where about 20 moles oxygen/mole protein was taken up; beyond this value no further changes were observed. These physical changes appear to bear some relation to a decrease of the tryptophan content of the protein. If it is assumed that the observed increase in viscosity is due to aggregation of a type similar to that produced by pH denaturation, one would expect that this increase would be eliminated if the native protein were pH-denatured prior to irradiation. The fact, however, that pH-denatured β-lactoglobulin showed an additional increase in viscosity during irradiation indicates that the changes in viscosity observed during irradiation of the native protein were different from that brought about by pH-denaturation.

The ultraviolet absorption spectrum of β-lactoglobulin during photooxidation shows that the absorption maximum at 279 mμ, which is chiefly due to the aromatic nucleus of tryptophan and tyrosine, was eliminated. The new absorption maximum formed around 260 mμ during the photoöxidation resembles the absorption behavior of dehydropeptides described by Greenstein (28). The possible unmasking of the benzene nucleus of tryptophan, discussed in our previous paper (2), may also be a factor in the changes in absorption.

The photochemical action of methylene blue on β-lactoglobulin does not involve hydrolysis of the peptide bonds, but rather an increase in molecular weight, as indicated by the light-scattering method and supported by solubility and viscometric studies.

Acknowledgments

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Summary

Optimal conditions for the photochemical action of methylene blue on β-lactoglobulin were studied.

It was shown that the first points of attack during photoöxidation of β-lactoglobulin are the aromatic nuclei of the histidine and tryptophan residues.

Tyrosine was photoöxidized only after the complete oxidation of histidine and tryptophan took place in the protein molecule.

A gradual decrease in the cystine content of the protein occurred during photoöxidation, but methionine was not affected.

A decrease in solubility and an increase in viscosity appeared to be associated with the decrease of the tryptophan content of β-lactoglobulin during photoöxidation.

At 20 moles oxygen uptake/mole β-lactoglobulin, the molecular weight increased to 147,000.

Photochemical action of methylene blue on β-lactoglobulin did not involve hydrolysis of the peptide bonds.

Photochemical action of methylene blue on lysozyme, insulin, salmine, and gelatin was studied.

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