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SUMMARY

β -Casein was readily photooxidized at pH 8.6 and 37 C with the reduction or disappearance of histidine, tryptophan, and tyrosine, in the order given. β -Casein oxidized to the extent of 18 moles of O₂ per 30,000 g casein was no longer precipitated by calcium chloride. The binding of calcium ions to β -casein was changed little or none by photooxidation. Photooxidation led to changes in ultraviolet absorption and a reduction in electrophoretic mobility in starch-gel-urea.

Photooxidation (oxidation catalyzed by light and a redox compound such as methylene blue) gives a limited, relatively specific oxidation of a protein under mild conditions of pH and temperature without breaking the peptide chain. Weil et al. (7, 9) have shown that the phenomenon is limited to the oxidation of tryptophan, histidine, tyrosine, methionine, and cystine. Weil et al. (8), in studies of lysozyme and subsequently in studies of other enzymes, have shown the value of this technique for revealing the amino acid side groups required for specific activity of enzymes.

Photooxidation studies of the caseins have been initiated (13) to determine whether this technique can reveal the amino acid side groups involved in the specific association between α_s - and κ -caseins resulting in a complex that does not precipitate with calcium ions (12). α_s -Casein alone precipitates with calcium ions. β -Casein, which also precipitates with calcium ions at room temperature, likewise associates with κ -casein to form a complex that does not precipitate with calcium ions (14). In the course of these studies, it has been found that photooxidation of α_s - and β -caseins makes these proteins less sensitive to precipitation with calcium ions. The present paper reports the photooxidation of β -casein and the changes that occur in its precipitation with calcium ions, changes in its amino acid components, and some of the accompanying physical and chemical changes.

MATERIALS AND METHODS

β -Casein. The β -casein was prepared by L. W. Nauman, III, by fractionation in urea (2). Starch-gel-urea electrophoresis showed a major band, the typical β -casein band, and a

trace of slower-moving material. This β -casein, prepared from commercial pooled milk obtained in the Philadelphia area, was examined for genetic type (1) and found to be Type A. Whole acid precipitated casein from the pooled milk contained predominantly β -casein of Type A, with a small amount of Type B. The latter was lost during the purification (R. F. Peterson and L. W. Nauman, III, unpublished studies).

Photooxidation. Photooxidation was studied manometrically in Warburg flasks in a rectangular water bath (12 by 28 in.) with glass sides, and a shaker for the flasks. The flasks were illuminated by a band of four 150-w lamps placed at the side (2½ in. from the glass wall) of the bath. The light was directed into the flasks by a 45-degree-angle brass-backed, ferrotype mirror placed 3 in. below the flasks in the bath. For the photooxidation each flask contained 2 ml of 1% β -casein in sodium borate buffer, pH 8.6 (46.6 g of boric acid and 10.0 g NaOH per liter); 0.4 ml of 20% KOH was placed in the center well with a 5- by 5-mm piece of Whatman No. 40 paper rolled into a cylinder; 0.5 ml of 0.02% methylene blue was placed in the side arm. (When the oxidation exceeded 12.5 moles O₂ per 30,000 g of casein only 1 ml of casein was used, with correspondingly smaller amounts of methylene blue.) The oxidation was carried out at 37.4 C in the presence of air. The methylene blue was tipped in from the side arm and the oxidation followed manometrically. The reaction was stopped by turning off the light. The methylene blue was immediately adsorbed onto IRC-50 resin (NH₄⁺ form), which was subsequently removed by centrifugation. This resin effectively removed methylene blue with little loss of protein. As photooxidation proceeded, however, traces of a greenish-blue derivative of methylene blue appeared which could not be removed. Other adsorbents recommended for methylene blue

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were also ineffective. The casein solutions after treatment with resin were dialyzed free of salts and freeze-dried.

Starch-gel-urea electrophoresis. Electrophoresis was performed by J. H. Custer of this laboratory in a horizontal type cell in 7 M urea, in borate buffer at pH 8.6, as described by Wake and Baldwin (6).

Ultraviolet absorption. The light absorption between 240 to 320 $m\mu$ of the β -casein solutions was determined in a Beckman DU spectrophotometer in a 1-cm cell. The caseins were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, centrifuged to remove a slight turbidity and read at the wavelengths indicated (see Figure 2) with the phosphate buffer in the reference cell. The N content of the solutions was determined with the Nessler reagent after digestion with sulfuric acid, and the factor 6.52 was used to convert weight of N to β -casein.

Amino acid analysis was done with an automatic amino acid analyzer on 24 and 72-hr HCl hydrolysates. Tryptophan was determined by the method of Spies and Chambers (4).

Precipitation of β -casein by calcium chloride. The precipitation experiments were done in 15-ml centrifuge tubes with a final volume of 5.0 ml. β -Casein (1.5 ml of a 0.5% solution at pH 7.5 to 7.8; after addition of CaCl_2 the final pH value was about 7.0), the required amount of water, and finally 0.1 M CaCl_2 were added to give the desired experimental concentrations.

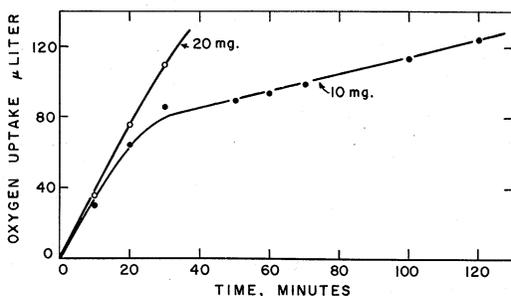


FIG. 1. Photooxidation of β -casein: uptake of O_2 in microliters with time per 10 mg (●—●) and per 20 mg (○—○) of β -casein.

The tubes were placed at 30 degrees for 15 min, and finally centrifuged at about $3,000 \times g$ for 5 minutes. Samples of the supernatant solutions were withdrawn, diluted 1:5, and one drop 10 M NaOH added to clarify the solution and bring to pH 13. The β -casein remaining in solution was determined from the light absorption at 280 $m\mu$.

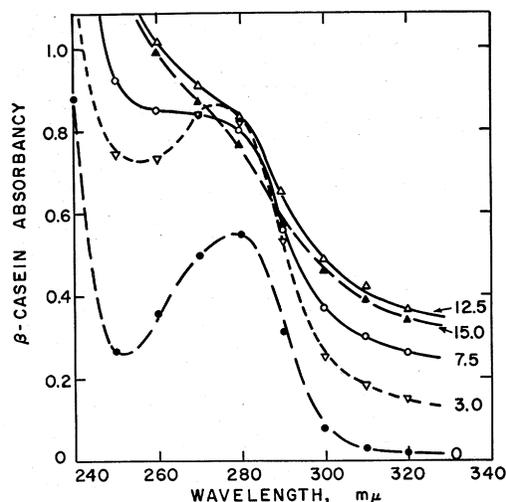


FIG. 2. Ultraviolet absorbance of β -casein photooxidized to various degrees. One milligram per milliliter at pH 7.0 in 1-cm cell. ● β -casein, ▽ β -casein photooxidized 3 moles O_2 per 30,000 g, ○ 7.5 moles O_2 , △ 12.5 moles O_2 , ▲ 15 moles O_2 . Results for 18 moles O_2 are almost identical with results shown by the 15 moles O_2 curve.

Calcium bound to casein. The β -casein solutions (1.0%) containing 0.005 M CaCl_2 and adjusted to pH 7.0 were ultrafiltered through dialysis tubing under pressure (5). The calcium in the ultrafiltrate, equivalent to unbound calcium, was determined by a spectrophotometric titration procedure (11).

RESULTS

Typical photooxidation-time curves for 20 and 10 mg of β -casein are shown in Figure 1. The 20-mg curve showed the same kind of change in slope as the 10-mg curve but at a correspondingly higher time and O_2 uptake. The photooxidations were stopped at desired degrees of oxidation and a number of runs pooled to give material for the following experiments: β -Caseins of the following degrees of oxidation (moles of O_2 per 30,000 g β -casein) were prepared: 3.0, 7.5, 12.5, and 18.0. (The 20 mg β -casein, Figure 1, terminated at the last experimental point shown, was oxidized to 7.5 moles O_2 per 30,000 g β -casein. The point at which the change in rate began in the 10 mg curve (60 μ l) corresponded to 8.0 mole of O_2 per 30,000 g β -casein.

The ultraviolet absorption curves at pH 7 for β -casein before and after various degrees of photooxidation are shown in Figure 2. The marked feature in these curves is the increase in absorption at 250 to 260 $m\mu$, so that when

the oxidation reaches 12.5 moles O_2 per 30,000 g casein, no minimum is apparent.

The decrease in the amino acids in β -casein sensitive to photooxidation is shown in Figure 3.

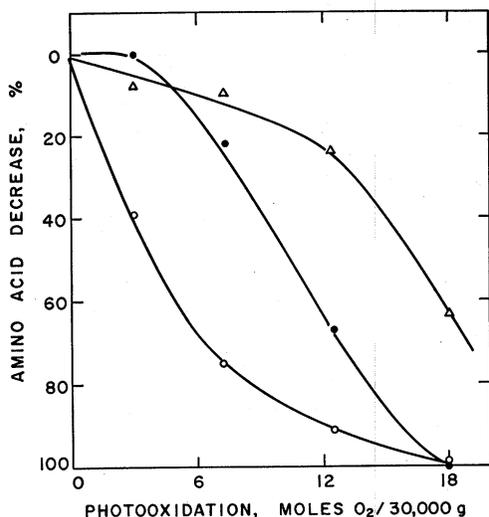


FIG. 3. Compositional change in β -casein on photooxidation (per cent decrease in particular amino acids). \circ Histidine, \bullet Tryptophan, Δ Tyrosine.

The starch-gel-urea electrophoresis patterns obtained at pH 8.6 of the β -casein and the photooxidized β -caseins are shown in Figure 4.

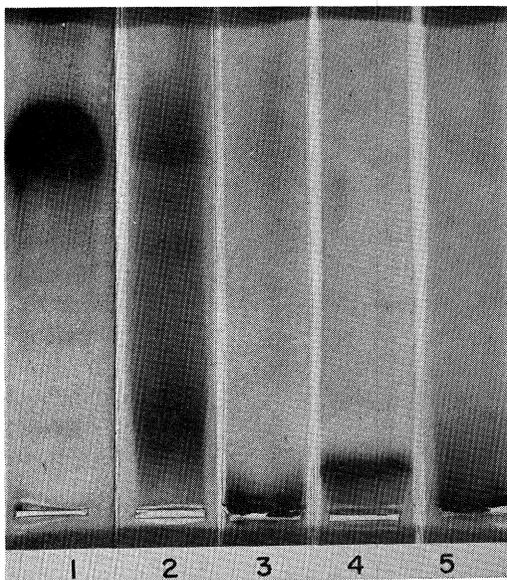


FIG. 4. Starch-gel-urea electrophoresis of β -casein and photooxidized β -caseins at pH 8.6. 1) β -Casein, (2-5) photooxidized β -caseins in order of oxidation (3, 7.5, 12.5, and 18 moles O_2 per 30,000 g β -casein).

The lowest degree of oxidation (3 moles O_2 per 30,000 g) pattern shows a transition stage in the changes that occur on photooxidation but at higher degrees of oxidation very little movement occurs in the gel on electrophoresis.

The precipitation of β -casein and the oxidized β -caseins by calcium chloride is shown in Figure 5. There is a marked decrease in the

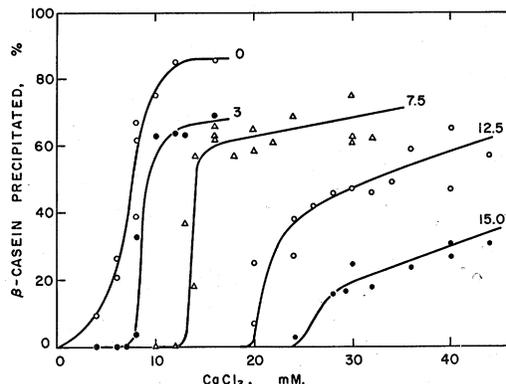


FIG. 5. Precipitation of β -casein and photooxidized β -caseins by calcium chloride at pH 7.0 and 30 C. The numbers by each curve represent the degree of oxidation as moles of O_2 per 30,000 g β -casein.

precipitation with increase in the degree of oxidation. β -Casein oxidized to 18 moles O_2 per 30,000 g casein was not precipitated by 30 mM $CaCl_2$, nor did it precipitate with a 160 mM concentration.

Some studies have been done of the binding of calcium to β -casein and photooxidized β -casein at pH 7.0. Values of 21 to 27 moles of calcium per 10^5 g β -casein were obtained for β -casein, similar to values measured by equilibrium dialysis (3). Values for the photooxidized β -caseins were within the same range except for the β -casein oxidized to 18 moles of O_2 per 30,000 g. In this instance the calcium-bound value was 15.

DISCUSSION

A characteristic feature of the photooxidation rate curves (Figure 1) is the marked change in rate that occurs at an intermediate degree of oxidation. In the 10 mg β -casein curve in Figure 1 the change in rate begins at an O_2 uptake of about 60 μ l, corresponding to 8.0 moles of O_2 per 30,000 g of β -casein. Weil et al. (9) have found that 1 mole of O_2 is required for the oxidation of 1 mole of histidine and 4 moles for the oxidation of tryptophan. With 6 moles of histidine and 1.25 moles of tryptophan per 30,000 g of β -casein, the above

data and the amino acid changes shown in Figure 3 suggest that the change in rate occurs when the oxidation of the histidine is approaching completion. The oxidation of the tryptophan and the tyrosine continues at a considerably slower rate.

The characteristic feature of the light absorption curves of photooxidized β -caseins is the marked increase in absorption at about 255 $m\mu$. These changes in absorption suggest that the aromatic amino acids are being oxidized, particularly histidine (10), and the analytical results in Figure 3 show that this is the case. Changes in other amino acids did not occur. The histidine and tyrosine were determined by column chromatography. Only small amounts of new components were observed, not sufficient to account for the oxidized derivatives. Considerably more humin is found on hydrolysis of the oxidized β -caseins than with the untreated β -casein and presumably some of the oxidized amino acids are lost in humin formation.

Results of starch-gel-urea electrophoresis (Figure 4) at pH 8.6 suggest that photooxidation may have led to some aggregation of the β -casein. With an O_2 uptake of 3 moles per 30,000 g of β -casein the pattern shows a transition stage in the changes that occur on photooxidation. At higher degrees of oxidation very little movement occurs in the gels on electrophoresis, presumably because of aggregation of the oxidized β -casein to a size that does not readily pass through the gel. α_s -Casein after photooxidation has a slightly greater electrophoretic mobility in starch-gel-urea. Photooxidized κ -casein, on the other hand, behaves like β -casein on electrophoresis in starch-gel-urea but on cellulose acetate, where electrophoresis is on the surface, the mobility is slightly greater than that of the untreated κ -casein. The photooxidized caseins when dried at pH values above 7 are readily soluble in water or buffers.

The most striking change in the properties of the β -casein after photooxidation is its poor precipitation with calcium chloride. The way in which this changes with increase in photooxidation is shown in Figure 5. β -Casein oxidized to 18 moles of O_2 per 30,000 g did not precipitate even with 160 mM concentration of calcium chloride. The explanation of this change is not apparent. An aggregated casein might be expected to precipitate more readily with calcium chloride. It was considered that oxidation of amino acid side groups might lead

to a casein with lessened ability to bind calcium. The binding studies show that this does not occur, or occurs only to a moderate degree at the extreme of oxidation. Hydrophilic groups such as $-OH$ may have been formed by photooxidation and so lead to enhanced solubility of the calcium salts that are formed irrespective of oxidation.

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