SPECTROPHOTOMETRIC OBSERVATION OF THE ALKALINE HYDROLYSIS OF PROTEIN DISULFIDE BONDS

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In the spectrophotometric titration of the phenolic groups of ovomucoid, a time-dependent increase in ultraviolet absorption is observed at high pH (Donovan, 1967). The present communication shows that the pseudo-first order rate constant for the absorption increase is proportional to the hydroxide ion concentration. The absorption change resembles the absorption of sulfhydryl ion, which is produced at the same rate as the absorption spectrum changes. The stoichiometry is consistent with S-S splitting of the disulfide bonds.

EXPERIMENTAL.[1] Absorption spectra in water or in 0.25 M KCl were measured at 22° with a Cary Model 15 spectrophotometer. Ovomucoid concentration was 4×10^{-6} to 1×10^{-5} M for spectrophotometric experiments. For experiments in which sulfhydryl production was measured at 22°, aliquots of reaction mixture, 2×10^{-4} M in ovomucoid, were added to Ellman's reagent in 1 M phosphate buffer, pH 8. Absorption spectra of the resulting solutions measured between 310 and 600 mμ were identical to those produced by dithiothreitol (Cleland, 1964). Sulfhydryl concentration was calculated from the absorption

[1] Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

change at 410 or 412 mμ (Ellman, 1959). The ovomucoid purification procedure has been described elsewhere (Donovan, 1967). Deionized water was used throughout. Removal of dissolved oxygen by degassing the solution before addition of alkali had no apparent effect. The EDTA and KOH used were ACS Reagent grade.

RESULTS. The change in the spectrum of ovomucoid observed immediately upon addition of alkali (Fig. 1A) results from the ionization of phenolic groups, since the difference spectrum has the characteristic peaks near 295 and 242 mμ. The time-dependent increase in absorption is then observed. The difference between the spectrum at infinite time and the spectrum extrapolated to zero time after addition of KOH is shown in Fig. 1B. Between 230 and 260 mμ, the absorption difference resembles the absorption of sulfhydryl ion. Absorption changes in the 260 - 300 mμ region are also observed.

Fig. 2 shows first order kinetic plots of both the change in absorption and the appearance of sulfhydryl groups. Fig. 3 shows the dependence of the pseudo-first order reaction rate constant, k', upon pH. These observations are consistent with: $d(\text{Products})/dt = k\ (OH^-)(\text{Protein})$, where $k' = k\ (OH^-)$. The bimolecular rate constant, k, is 0.010 liters mole^{-1} sec^{-1} at 22°.

Ovomucoid contains disulfide bonds, but no sulfhydryl groups (Lineweaver and Murray, 1947). When ovomucoid is dissolved in 6 M guanidine hydrochloride at pH 8, and Ellman's reagent added, no sulfhydryl groups are detected (< 0.1 SH/molecule). There are either 9 (Stevens and Feeney, 1963) or 10 (Deutsch and Morton, 1961) disulfide bonds per molecule of ovomucoid (30,000 mw). Essentially all these disulfide bonds are split by alkali. At pH 13.5 to 13.7 and 22°, analyses using Ellman's reagent show that 12 to 13 SH groups are produced in the first 45 min. A slower production of material reducing Ellman's reagent follows. Calculations show that a total of 15 SH groups are produced at infinite time. Assuming the absorption change at 240 mμ is produced solely by sulfhydryl groups, its magnitude at higher pH is consistent with the formation of 13 to 15 sulfhydryl groups.

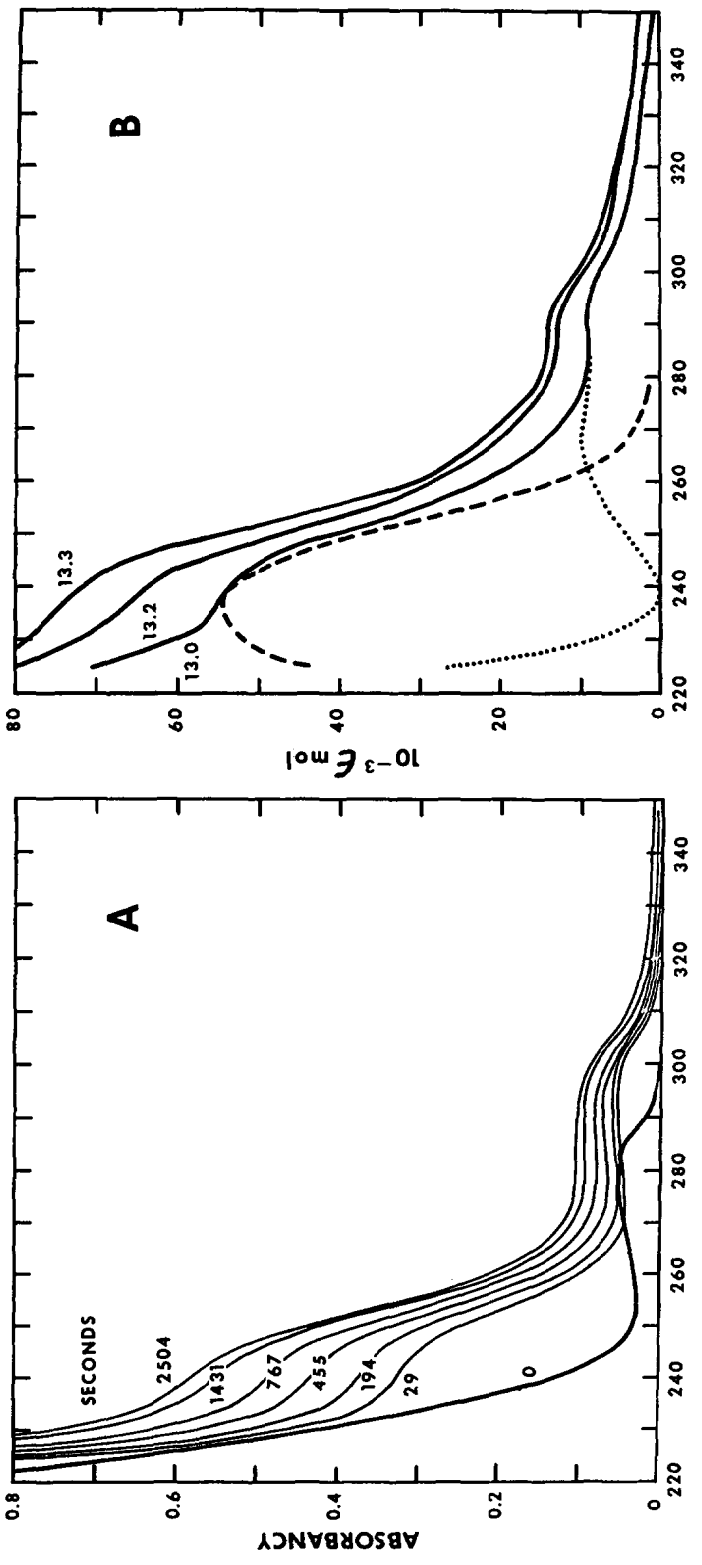

Figure 1. A, absorption spectra of a 4.2×10^{-6} M solution of ovomucoid, before (0) and after addition of KOH to bring the pH to 13.0. The time of the absorption scan (to shorter wavelength) was determined at 240 mμ. The large absorption change occurring in the first 30 sec is produced by tyrosine ionization. B, the total change in absorption per mole of ovomucoid, not including tyrosine ionization, observed at three pH values. These absorption spectra are the differences between the absorption spectrum at infinite time and the absorption spectrum back-extrapolated to zero time after addition of alkali. The pH 13.0 curve is approximately the difference between the two curves labeled 2504 sec and 29 sec in Fig. 1A. The dashed curve is an absorption spectrum of 10 moles of sulfhydryl ion (per mole of ovomucoid), calculated using n-butylmercaptan in 1 N NaOH as model (Noda, et al., 1953). The dotted curve is the residual spectrum obtained when the dashed curve is subtracted from the pH 13.0 spectrum. The small absorption decrease (not shown) resulting from loss of disulfide bonds is neglected here.

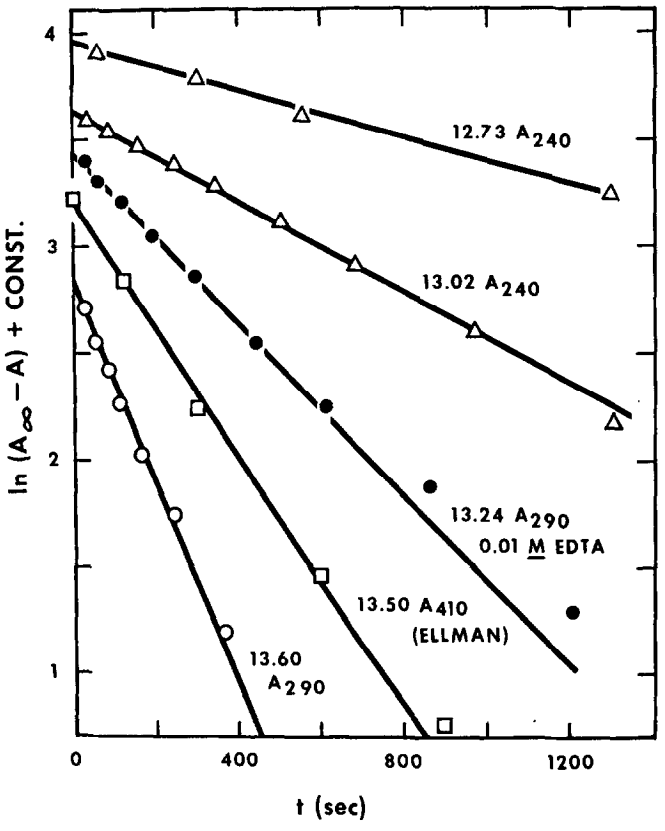

Figure 2. First order kinetic plots of absorption changes and production of sulfhydryl groups. Absorption changes observed at 240 mμ are shown as triangles; at 290 mμ, by circles. Filled circles: measurements made with 0.01 M EDTA present; squares: production of sulfhydryl groups measured using Ellman's reagent. The pH of the reaction mixture is shown for each curve.

The absorption observed above 270 mμ must be produced by some other product. Fig. 3 shows that the kinetics observed at 290 mμ are identical with those observed at 240 mμ. The nature of this product has not been demonstrated. Amino acid analysis of an acid hydrolysate of a sample of ovomucoid exposed to alkali showed only one-fifth of the usual amount of half-cystine, and a decrease in the amount of lysine (from 14.0 to 11.4 residues per mole) which could be quantitatively accounted for by the amount of a new peak eluted in the position expected for lysinoalanine (Bohak, 1964).

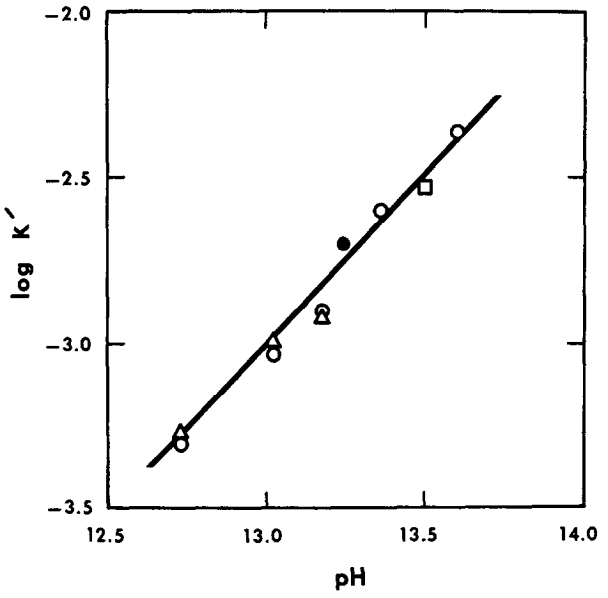

Figure 3. Dependence of the pseudo-first order reaction rate constant (sec^{-1}) upon pH. Symbols are the same as in Fig. 2. The line drawn through the points has a slope of unity.

DISCUSSION. The results are consistent with the reaction scheme (Challenger and Rawlings, 1937; Schöberl and Hornung, 1938) in which three sulfhydryl groups are produced for every two disulfide bonds split by hydroxide ion, if step (1) is rate-limiting:

$$RS\text{-}SR + OH^- = RS^- + RSOH \quad (1)$$

$$2\ RSOH = RSH + RSO_2H \quad (2)$$

$$2\ RS\text{-}SR + 4\ OH^- = 3\ RS^- + RSO_2^- + 2\ H_2O \quad (3)$$

Wronski (1963) has shown that this stoichiometry and kinetic behavior is true for the hydrolysis of cystine in 2 N NaOH at 60°. The results of Rivett et al. (1965) also demonstrate that S-S fission of cystine occurs in strong alkali, but that C-S fission may take place at pH 11. Gawron and Odstrchel (1967) show that C-S fission accompanied by β-elimination accounts for the hydrolysis of amino- and carboxyl-substituted cystine at 90° near pH 10. Products are a persulfide and dehydroalanine. C-S fission could account for

the lysinoalanine observed in the case of ovomucoid, but cysteine itself may also undergo β-elimination at high pH. Coincidentally, the absorption spectrum of dehydroalanine (Carter and Greenstein, 1946) closely resembles that of sulfhydryl ion, so that the absorption changes near 240 mμ cannot be used to distinguish between the two hydrolysis mechanisms.

The disulfide bonds of ovomucoid appear to be completely hydrolyzed under the present conditions, although the amino acid cystine, when similarly treated, shows no absorption change. Steric strain may be responsible. Wolfram (1965) has shown enhanced reactivity of disulfide bonds in mechanically strained keratin. Ovomucoid, with an isoelectric point near pH 4 (Bier et al., 1953), may be subject to strong electrostatic forces tending to expand the molecule at high pH. Although disulfide bonds readily undergo S-S fission in the presence of metal ions (Cecil and McPhee, 1957; Klotz and Campbell, 1962), the addition of EDTA does not affect the reaction rate (Fig. 3). Disulfide bonds attached to aromatic rings undergo fission readily; Klotz and Campbell report a substituted diphenyl disulfide split by hydroxide ion at pH 10.8. Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid), showed absorption changes in alkali very similar to those observed upon reduction. The absorption change at 412 mμ was 0.73 of that observed upon reduction, suggestive of S-S fission.

This absorption change in alkali occurs with other proteins. Changes in the spectrum of RNAse at pH 13 occur within 30 min at 22°. Bohak (1964) showed that five half-cystine residues disappeared from RNAse in 15 min at 40° in 0.2 N NaOH. As with ovomucoid, difference spectra of RNAse at higher pH do not pass through the isosbestic points near 272 and 278 mμ, characteristic of phenolic ionization.

When spectrophotometric titrations of phenolic groups in proteins are carried out, the entire spectrum or difference spectrum should be examined to ensure that other spectral changes, such as those presented here, are not incorrectly ascribed to tyrosine ionization.

The magnitude of the bimolecular rate constant for the alkaline fission of the disulfide bonds of ovomucoid suggests that hydrolysis of disulfide bonds by hydroxide ion is responsible for the thinning of egg white of eggs held in storage. This aspect is currently under study in this laboratory.

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