Alkaline Hydrolysis of the Disulfide Bonds of Ovomucoid and of Low Molecular Weight Aliphatic and Aromatic Disulfides*

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ABSTRACT: The rate of reaction of hydroxide ion with the disulfide bonds of ovomucoid from chicken egg white, 2,2′-dithiodiethanol, 3,3′-dithiodipropionic acid, and 5,5′-dithiobis-(2-nitrobenzoic acid) was determined as a function of temperature by following the appearance of thiol spectrophotometrically. Activation energies for ovomucoid and the aliphatic disulfide compounds were all 19 kcal/mole, but that of the aromatic disulfide was 15 kcal/mole. Ionic strength had a marked effect on the rate of hydrolysis of ovomucoid, but no apparent effect on the activation energy. Amino acid analyses of ovomucoid carried out as a function of time of alkaline hydrolysis showed that loss of cystine occurred at the same rate as production of thiol, and that some lysinoalanine was produced.

Conformational changes of ovomucoid occur at the same rate as hydrolysis of its disulfide bonds.

Use of the change in ultraviolet absorption near 240 mμ to measure the rate of alkaline hydrolysis of disulfide bonds (Donovan, 1967a) allows direct measurement of rates of reaction in the solution in which the reaction is progressing. This is in contrast to kinetic methods previously used, in which samples were taken from the reaction mixture and treated chemically to convert reaction products into other compounds which can be quantitated conveniently. However, direct measurement of the change in the absorption does not, in itself, ordinarily identify either the reactants or products, so that some chemical methods must be used to interpret the absorption changes. In the experiments described here, the increase in absorption in the 240-mμ region produced by alkali treatment of disulfides has the absorption spectrum characteristic of the thiol anion, RS^-. In addition, the amount of absorption change is quantitatively in accord with the amount of thiol determined by treatment of aliquots of the reaction mixture with Ellman's (1959) reagent under mild conditions (a few minutes at pH 8, room temperature).

The mechanism of cleavage of disulfide bonds by alkali remains unclear despite a large number of studies of proteins and of low molecular weight model compounds (for reviews, see Cecil and McPhee, 1959, and Danehy, 1966). Proposed initial steps of the reaction include: (1) direct nucleophilic attack of hydroxide ion on one of the sulfur atoms of the disulfide bond; (2) elimination reactions in which hydroxide ion removes a proton: (a) from the carbon atom α to the disulfide bond (α elimination) and (b) from the carbon atom β to the disulfide bond (β elimination). Proposed subsequent steps include cleavage of the covalent bond between carbon and sulfur atoms or between the two sulfur atoms of the disulfide bond. Experimental evidence supporting these different mechanisms has been presented (Swan, 1957; Danehy and Kreuz, 1961; Wronski, 1963; Bohak, 1964; Danehy and Hunter, 1967; Gawron and Odstrchel, 1967).

The present paper is an extension of studies of the hydrolysis of disulfide bonds of ovomucoid (Donovan, 1967a), and includes spectrophotometric studies of hydrolysis of model disulfides which have been previously studied by more classical techniques. In particular, activation energies, apparently never reported for disulfide-bond hydrolysis, have been determined. Some consideration has been given to the following questions particularly related to hydrolysis of disulfide bonds in ovomucoid. How is the rate of hydrolysis affected by the net charge of the protein and the ionic strength of the solvent? Must a conformational change of the protein take place *before* the disulfide bonds can be hydrolyzed? Does hydrolysis of disulfide bonds *produce* a conformational change in the protein?

Materials and Methods[1]

Ovomucoid was obtained from Worthington Biochemical Co., and purified by the method devised by J. G. Davis. Purification and characterization of this material is described in the accompanying paper (Davis *et al.*, 1971). The concentration of solutions prepared by dissolving freeze-dried ovomucoid in water, buffer, or salt solution was determined by use of the absorption coefficient, $E^{1\%}_{1\ cm}$ 4.10 (Donovan, 1967b). Eastman Kodak White Label 2,2′-dihydroxyethane disulfide (2,2′-dithiodiethanol) was used without further purification. Dithiodipropionic acid was prepared from Eastman Kodak White Label 3-mercaptopropionic acid as described by Danehy and Kreuz (1961). Ellman's (1959) reagent 5,5′-dithiobis-(2-nitrobenzoic acid) was Aldrich Chemical Co. (lot no. 092551).

Absorption spectra were recorded on a Cary Model 15 spectrophotometer equipped with IP 28 photomultipliers and thermostatable cell compartment. The spectrophotometer was carefully checked for drift of base-line absorption with time. Such drift would have produced inaccurate optical density readings at infinite time in kinetic runs. Temperature control was obtained by circulating water from a Haake circulator through the thermostatable cell holder. Temperatures (±0.1°) were measured inside the 1-cm absorption cell,

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equilibrated at temperature, by use of a calibrated thermometer.

Measurements of optical rotation were made with the Perkin-Elmer Model 141 polarimeter. Viscosity measurements were made as described previously (Donovan, 1967b). The molecular weight of ovomucoid was assumed to be 29,600, and the mean residue weight, $\bar{M}$, 149. Production of thiol was measured chemically by use of Ellman's (1959) reagent as previously described (Donovan, 1967a).

Kinetics were measured spectrophotometrically in two ways. (1) A preliminary method, in which the entire absorption spectrum of the reaction mixture was scanned at intervals of time so that all absorption changes could be observed. In all cases, differences between spectra obtained at different times showed that only one type of absorption change occurred throughout the course of the reaction. (2) After the nature of the change in the spectrum was ascertained, kinetics were measured by fixing the wavelength and reading absorption as a function of time—either (a) until the change in absorption ceased entirely (t_∞ measurement), (b) most of the change (>90%) was completed, or (c) the initial change in absorption became significantly nonlinear (initial velocity method). Procedure c was used for the reactions of the two aliphatic disulfides, since the rates of reaction were very slow. For these compounds, concentrations were calculated using ϵ_{240} 300 for disulfide (Rosenthal and Oster, 1961) and ϵ_{240} 5000 for sulfhydryl ion (Noda *et al.*, 1953). When data obtained by method a were subjected to linear least-squares analysis for first-order kinetics, the parameters obtained (rate constant, total change in absorption) proved to be no more precise than those obtained by subjecting the data obtained by method b to a linear least-squares analysis for first-order kinetics by means of the Guggenheim (1926) calculation, for which A_∞ is not required. The probable reason for this is the development of slight turbidity changes in the solutions. These changes can produce relatively large errors when the rate of change of absorption is very small at the end of the reaction. Accordingly, most kinetic data for ovomucoid were obtained by method b. The standard error in the rate constant was usually less than 2% of the rate constant. When method c was used to follow the kinetics of hydrolysis of ovomucoid, at least one reaction of the series was checked by method b.

For consistency, in the absence of convincing evidence for stoichiometry, the rate equation

$$d(RS^-)/dt = k(S\text{–}S)(OH^-) \quad (1)$$

was integrated using the stoichiometry: 1S–S = 1RS$^-$, and the second-order rate constants are reported on this basis. Once the stoichiometry of the reactions has been definitely determined, the correct rate constant can be obtained by a trivial calculation. The temperature dependence of the rate constants was expressed in the form

$$k = pZ' \exp(-E_a/RT) \quad (2)$$

where k is the second-order rate constant.

Amino acid analyses were carried out as described by Davis *et al.* (1969) except that only 20-hr HCl hydrolyses were carried out, and no corrections for destruction of amino acids or hexosamine upon HCl hydrolysis were made. Analyses are reported as ratio of amino acid to aspartic acid, which remained constant on alkaline hydrolysis. Use of glycine or glutamic acid as reference amino acids gave equivalent results.

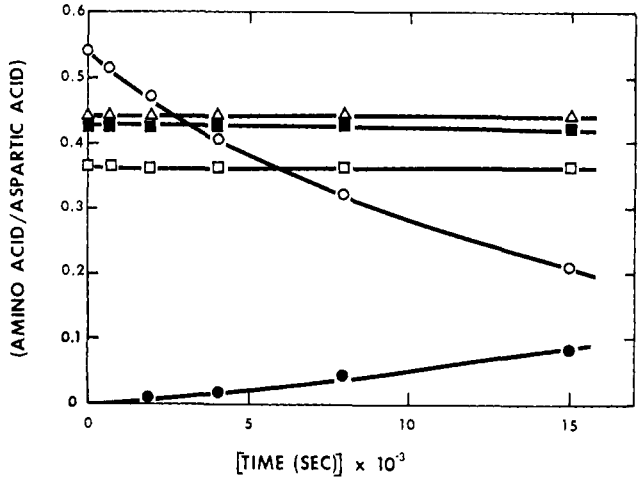


FIGURE 1: Amino acid composition of ovomucoid as a function of time of exposure to pH 11.8 at 30.4°. Analyses of 20-hr hydrolysates (6 N HCl, 110°) for each amino acid are expressed relative to aspartic acid content, which was observed to remain constant with time: (○) half-cystine, (△) threonine, (□) serine, (●) lysinoalanine, and (■) lysine plus lysinoalanine.

Results

Ovomucoid. The previous work (Donovan, 1967a) showed that hydrolysis of the disulfide bonds in ovomucoid (which contains no cysteine) is first order in hydroxide ion, is not metal ion catalyzed, and gives a thiol as a product. Thiol was liberated with the same rate constant as the change in absorption near 240 mμ (Donovan, 1967a), indicating that absorption change at 240 mμ could be used as a measure of thiol production. It appeared expedient also to determine the rate as measured by decrease in disulfide content of ovomucoid. The results of amino acid analyses of 20-hr hydrolysates of samples taken from a thermostated container as a function of time of hydrolysis are given in Figure 1. An analysis of the kinetics of the loss of half-cystine by the Guggenheim procedure indicated that all half-cystine would be used up at infinite time in a first-order reaction, in agreement with the the earlier result (Donovan, 1967a). On this basis, the rate constant for loss of cystine calculated from the data shown in Figure 3 by linear least-squares fit to first-order kinetics was $6.40 \pm 0.10 \times 10^{-5}$ sec^{-1} at pH 11.81, 30.4°, ionic strength (I) = 0.06. This rate constant was compared to the rate constant obtained by change in A_{240} *vs.* time at essentially the same ionic strength and temperature, but at slightly different pH (12.16) by dividing both first-order rate constants by hydroxide ion activity. The second-order rate constants so obtained were 5.3×10^{-3} M^{-1} sec^{-1} as measured for loss of cystine and 5.6×10^{-3} M^{-1} sec^{-1} as measured by increase in absorption at 240 mμ. The substantial agreement between these rate constants indicates that thiol appears at the same rate as disulfide disappears.

Figure 1 shows that the amount of lysinoalanine (Bohak, 1964) formed in 15,000 sec is about one-quarter of the amount of half-cystine reacted at this time. The ratio of the amount of lysinoalanine formed to amount of half-cystine lost increases with time, as expected if lysinoalanine is formed from an intermediate dehydroalanine. The complete amino acid

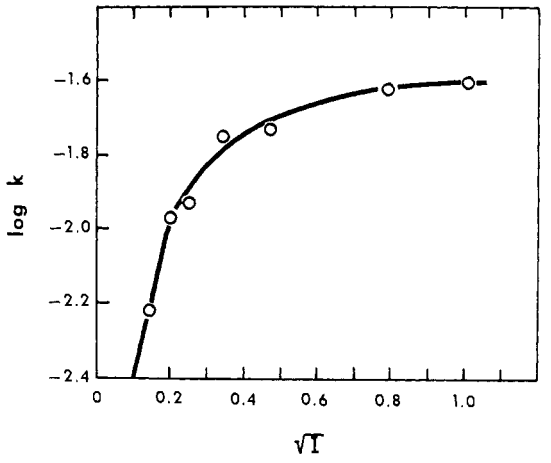


FIGURE 2: Ionic strength dependence of the second-order rate constant for the hydrolysis of the disulfide bonds of ovomucoid, pH 12.16, 30.8°.

analyses showed that the content of other amino acids was not affected, as Bohak (1964) has observed. However, at higher temperatures, Adams (1965) observed loss of threonine. Figure 1 shows that no loss of threonine or serine occurred.

Both ovomucoid and hydroxide ion bear negative charges at alkaline pH. Ionic strength affects the reaction velocity (Figure 2). At the lowest ionic strengths at which measurements could be conveniently made, the limiting slope of the log k *vs.* $(I)^{1/2}$ plot is approximately 4, much less than the product of charges, Z_{OH}-$Z_{ovomucoid}$, of the Debye–Hückel-limiting law, estimated to be of the order of 30 at this pH. It is probable that salt binding or inapplicability of the Debye–Hückel-limiting law due to insufficiently low ionic strength is responsible for the small value of the slope observed.

The temperature dependence of the second-order rate constant for hydrolysis of disulfide bonds is shown in Figure 3. The activation energy for ovomucoid appears not to be a function of ionic strength.

Hydrolysis of disulfide bonds in ovomucoid occurs at a considerably faster rate than hydrolysis of the disulfide bonds in the aliphatic disulfides tested. Ovomucoid appears to be a typical protein in this respect, since rates of alkaline hydrolysis of disulfide bonds of ribonuclease (Bohak, 1964), lysozyme, and ovomucin (unpublished data from this laboratory) are of the same order of magnitude. Cecil and McPhee (1959) suggest that, on the basis of the β-elimination mechanism, the electron-attracting peptide bonds attached to the α-carbon atom of the amino acid residue should make the rate of hydrolysis of the protein disulfide bond comparable to that of cystinephenylhydantoin or dialanylcystine dianhydride. This appears to be the case. Table V of Cecil and McPhee (1959) gives the relative rate (compared to cystine = 1) of these two compounds as 75, and the relative rate for hydrolysis of dithiodipropionic acid as 0.04. The difference in the logarithm of the rate constants is thus $\Delta(\log k) \simeq 3.3$. From Figure 3, $\Delta(\log k)$ between ovomucoid and 3,3′-dithiodipropionic acid is 3.25 under approximately equal conditions of pH and ionic strength, at the same temperature. Thus, the apparently high reactivity of the disulfide bonds of proteins is predictable upon the basis of the β-elimination mechanism. However, there is a remote possibility that hydrogen bonds, if present, between amide groups and sulfur atoms of the disulfide bonds (Donohue, 1969) might influence the rate of the hydrolysis reaction.

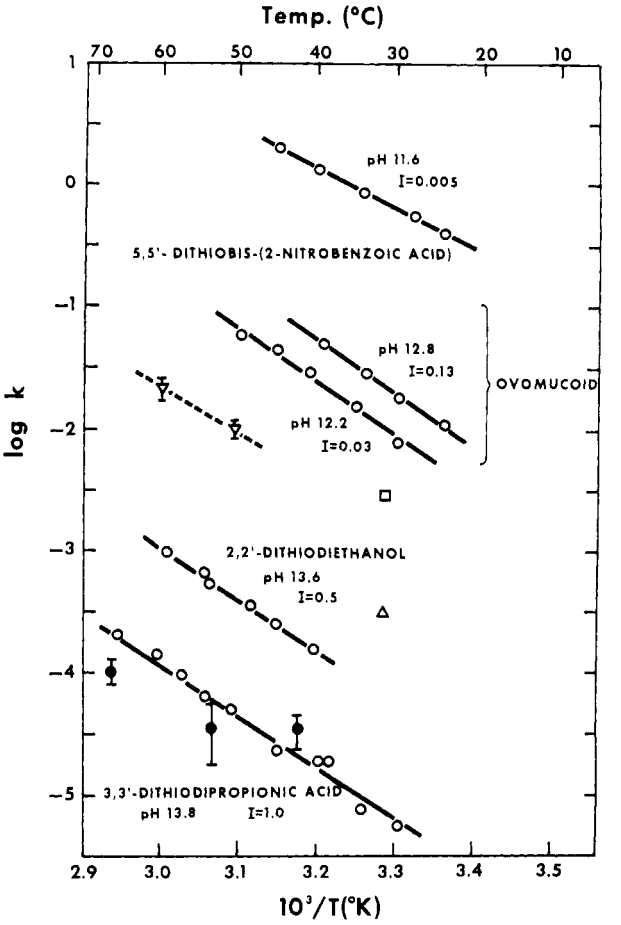


FIGURE 3: Temperature dependence of the second-order rate constants for alkaline hydrolysis of the disulfide bonds of ovomucoid and model compounds. Indicated values of pH and ionic strength were determined at room temperature. All solutions contained only KOH and sufficient KCl to maintain ionic strength. With the exception of 5,5′-dithiobis(2-nitrobenzoic acid), all rate constants determined by measurement of rate of change of A_{240} are represented by (○); by release of thiol ion, as measured by Ellman's reagent (●). The rate constants obtained by Gawron and Odstrchel (1967) for hydrolysis of dicarbobenzyloxycystinyldiglycine and the corresponding cystinyldialanine are represented by □ and △, respectively. The average rate constants for the alkaline hydrolysis of cystine at 50 and 60°, as well as the range of rate constants reported by Wronski (1963) are represented by ▽.

Aliphatic Disulfides. The temperature dependence of the alkaline hydrolysis of the disulfide bonds of 2,2′-dithiodiethanol and 3,3′-dithiodipropionic acid is shown in Figure 3. For these kinetic measurements, the pH was chosen so that the reaction proceeded rapidly enough that initial velocities could be obtained accurately within a period of about 1 hr.

The rate of formation of thiol from 3,3′-dithiodipropionic acid, as measured using Ellman's (1959) reagent (Figure 3) is the same, within experimental error, as the rate of formation of thiol calculated from the change in A_{240}. Differences between ultraviolet spectra taken at different time intervals were characteristic of thiol ion (Figure 4).

The second-order rate constants shown in Figure 3 were derived from the pseudo-first-order rate constants by division by hydroxide ion activity, or by division by both hydroxide ion activity and disulfide concentration when the initial rate only was measured. The rate of hydrolysis of the disulfide bond in 2,2′-dithiodiethanol observed here is about 50% greater than that reported by Danehy and Hunter (1967).

TABLE I: Second-Order Rate Constants, Activation Energies, and Frequency Factors for Hydrolysis of Disulfide Bonds.

Compound	pH	Ionic Strength	k (40°) (M^{-1} sec^{-1})	E_a (kcal/mole)	Log pZ'
Ovomucoid	12.8	0.13	5.8×10^{-2}	18.8 ± 0.5	11.7
Ovomucoid	12.1	0.03	3.1×10^{-2}	19.9 ± 1.1	12.3
2,2′-Dithiodiethanol	13.6	0.54	1.6×10^{-4}	18.8 ± 0.6	9.3
3,3′-Dithiodipropionic acid	13.8	1.00	1.9×10^{-5}	19.4 ± 0.8	8.8
5,5′-Dithiobis-(2-nitrobenzoic acid)	11.6	0.005	1.3	14.9 ± 0.3	10.6

They state that the rate of hydrolysis of 2,2′-dithiodiethanol is four- to fivefold faster than that of 3,3′-dithiodipropionic acid. This difference in rate may be due in part to the negative charge on the acid (which has COOH groups substituted for the OH groups of 2,2′-dithiodiethanol), since the disulfide bond of the ester is hydrolyzed at least three times as rapidly as the 3,3′-dithiodipropionic acid itself (Danehy and Hunter, 1967). We have not investigated the effect of ionic strength upon the rate of hydrolysis of 3,3′-dithiodipropionic acid, but the initial rate observed here is within a factor of two of the initial rate of hydrolysis for this compound derived from Table I of Danehy and Hunter (1967).

Aromatic Disulfides. The temperature dependence of the rate constant for hydrolysis of 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959), the only aromatic disulfide studied here, is given in Figure 3. The rate of hydrolysis was followed by the increase in absorption at 412 mμ. Figure 5 shows that the total change observed in A_{412} is 0.73 that obtained when dithiothreitol is added to completely convert the disulfide into thiol. This, as discussed previously (Donovan, 1967a), suggests that alkaline hydrolysis of this compound produces three thiol groups for every two disulfide bonds, in agreement with previous studies on aromatic and on some aliphatic disulfides (see Danehy and Hunter (1967) for earlier references).

A summary of the kinetic data in terms of activation energies and frequency factors is presented in Table I. A search of the literature did not reveal any determination of the activation energies of hydrolysis of disulfide bonds. However, Wronski (1963) reported rate constants at 50 and 60° (two temperatures only) for alkaline decomposition of cystine. These rate constants have been plotted in Figure 3. Rate constants reported by Gawron and Odstrchel (1967) for (β-elimination type) alkaline hydrolysis of cystine endopeptides at 90.5° (borate buffer) and at 31° (in NaOH) can be used to calculate activation energies of 21 kcal/mole for *N*,*N*′-dicarbobenzyloxy-L-cystinyldiglycine and 31 kcal/mole for the corresponding dialanine. These experiments, like Wronski's, were not designed to determine the activation energy, and it is doubtful whether an activation energy calculated from two constants obtained in different buffers over such a large temperature range is meaningful. The rate constants obtained at 31° have been plotted in Figure 3.

Conformational Studies of Ovomucoid. Physical measurements were carried out on solutions of ovomucoid before, after, and during the course of alkaline hydrolysis, to attempt to determine both (a) the relationship between conformation of the protein and the rate of hydrolysis of disulfide bonds and (b) the effect of hydrolysis of disulfide bonds on the conformational stability of the protein. Figure 6A shows the dependence of optical rotation on temperature for native ovomucoid and for ovomucoid after hydrolysis of its disulfide bonds. In *neither* case is there a sigmoid-shaped curve, indicative of cooperative conformational change. The direction of the change in rotation is the reverse of that observed for denaturing transitions.

Most of the difference in optical rotation at 25° between native and alkali-treated ovomucoid observed in Figure 6 is produced immediately upon addition of alkali (Figure 6B), and can thus be attributed to a conformational change produced by pH. Analysis of the slow increase in rotation which follows shows that it has the same time dependence as the change in A_{240}. The change in rotation with time is thus produced by splitting of the disulfide bond (Würz and Haurowitz, 1961; Tanford *et al.*, 1967), or by a conformational change in ovomucoid, or both. The direction of the change in rotation (increase) is that expected for hydrolysis of disulfide bonds (*i.e.*, conversion of cystine into half-cystine or cysteine). However, the amount of change in *molar* rotation divided by the number of disulfide bonds split ($\Delta[\phi]^{25°}_{365}/n_{S-S}$) is only about 260 (assuming all disulfide bonds split). Estimates of this quantity made by Tanford *et al.* (1967) in denaturing solvents, interpolated at 365 mμ

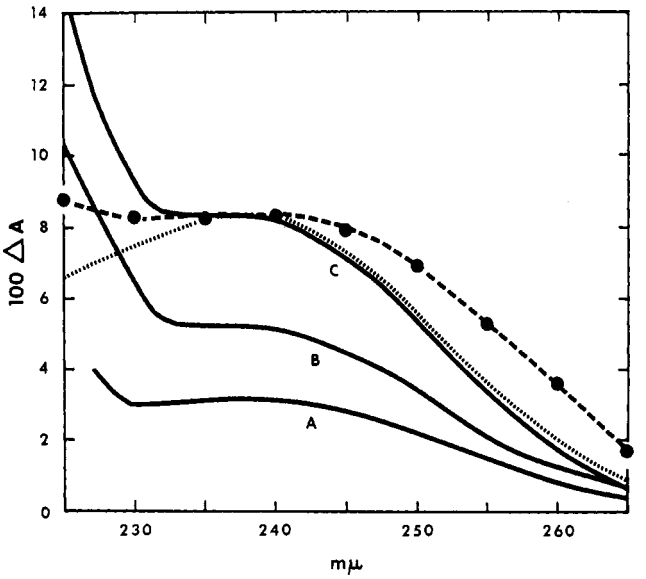


FIGURE 4: Changes in absorption on addition of 3,3′-dithiodipropionic acid to 1 M NaOH at 45°. Solid lines are differences in absorption of 1.6×10^{-4} M dithio acid observed between: (A) 35 min and 78 min, (B) 7 min and 78 min, and (C) 7 min and 145 min. Plotted on the same scale for comparison are the spectra of *n*-butylthiol anion, ·····, (Noda *et al.*, 1953) and glycyldehydroalanine, – – – –, (Carter and Greenstein, 1946).

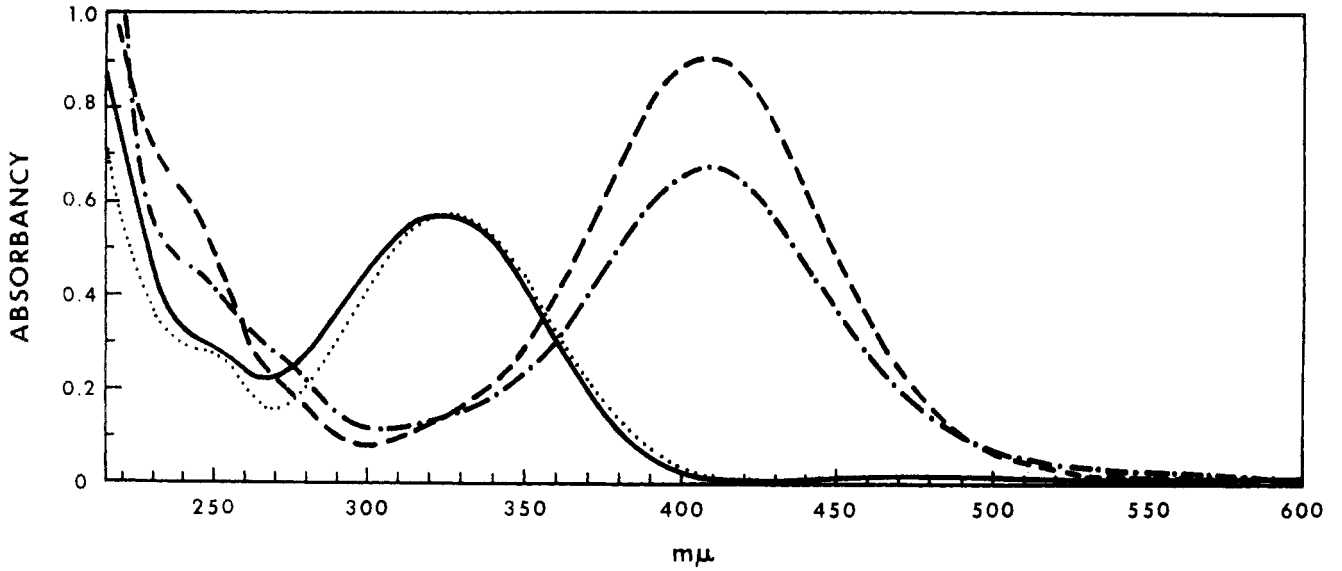


FIGURE 5: Spectra of 5,5′-dithiobis-(2-nitrobenzoic acid). At pH 8, ————; quantitatively converted into the corresponding thiol ion by addition of excess solid dithiothreitol at pH 8, – – –; spectrum of the un-ionized thiol produced by adding a small amount of concentrated HCl after addition of an excess of dithiothreitol, · · · · · · · · · ; fresh aliquot after completion of reaction of disulfide bonds with alkali, –·–·–·.

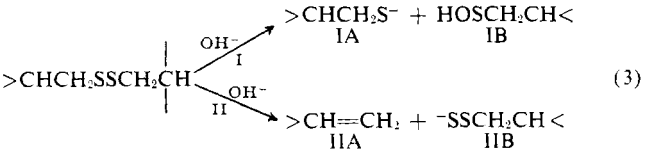
(the wavelength used here) average about 900. The most reasonable interpretation of this change in optical rotation thus appears to be an increase in rotation due to disulfide splitting superimposed on a decrease in rotation normally observed on denaturation (see below).

Measurements of viscosity of native ovomucoid show little change in η_{sp}/c with pH and ionic strength. In the pH range 10.9–11.1, variation of ionic strength from 0.1 to 0.9 showed no change in viscosity within experimental error. Values of η_{sp}/c at 10 mg/ml ranged from 5.2 to 5.6 ml per g. These viscosities are not very different from those observed at pH 4–5: $[\eta] = 5.5$ ml/g, but are smaller than the viscosity observed in acid, $[\eta] = 6.3$ ml/g at pH 2.1 (Donovan, 1967b).

After addition of alkali to raise the pH to 13.1, the viscosity of an ovomucoid solution (17 mg/ml) was measured as a function of time. The initial reduced viscosity (14 ml/g at 200 sec after mixing) was more than double that observed at pH 11, showing that a conformational change occurred before hydrolysis took place. The reduced viscosity increased progressively with time, becoming constant within experimental error at 28 ± 4 ml/g after 3000–5000 sec. An estimate of the pseudo-first-order rate constant was made from the value of $t_{1/2}$ (650 sec) for the rate of change in viscosity. The estimated second-order rate constant was in reasonably good accord with that determined from rate measurements of changes in the absorption spectrum: 1.7×10^{-2} M^{-1} sec^{-1} by viscosity change, 1.3×10^{-2} M^{-1} sec^{-1} by ΔA_{240} (23.6°, ionic strength 0.7). Thus, conformational changes take place upon hydrolysis of disulfide bonds.

Discussion

The results presented here demonstrate that the activation energy for hydrolysis of disulfide bonds in proteins and aliphatic disulfides is identical, suggesting a common rate-limiting step. As previously demonstrated for the alkaline hydrolysis of ovomucoid (Donovan, 1967a), thiol is produced in the alkaline decomposition of 3,3′-dithiodipropionic acid at the same rate as the change in absorption at 240 mμ occurs. The quantitative agreement between the change in A_{240} and amount of thiol liberated suggests that the appearance of thiol occurs in the rate-limiting step of alkaline hydrolysis, and that S–S fission takes place for both aliphatic disulfides and protein. The two mechanisms most commonly invoked to explain alkaline fission of disulfide bonds are



(3)

Reaction products I are those of the "classical" S–S split mechanism of Schöberl (1933). The sulfenic acid, IB, if formed, is unstable or highly nucleophilic (Kice and Cleveland, 1970) and probably undergoes further reaction with disulfide (Rivett *et al.*, 1965) or by dismutation to form thiol, RS^-, and sulfinic acid, RSO_2^-.

The data presented here, by Rivett *et al.* (1955) and Danehy and Hunter (1967) for the aliphatic disulfides, by Wronski (1963) for cystine, and for ovomucoid (Donovan, 1967a) appear to favor mechanism I. The product IA would have the ultraviolet absorption of thiol anion in alkaline solution (see Figure 4) and its reaction with Ellman's reagent would be quantitatively in agreement with the absorption change, as observed. The sulfinic or sulfonic acid formed from the sulfenic acid product IB should have little ultraviolet absorption near 230 mμ, in analogy with sulfites and sulfates (Buck *et al.*, 1954). Assuming decomposition into sulfinic acid, the net reaction would be

$$2RSSR + 4OH^- \longrightarrow 3RS^- + RSO_2^- + 2H_2O \qquad (4)$$

A larger proportion of thiol would result if the hypothetical sulfenic acid were converted into sulfonic acid. For alkaline decomposition of 2,2′-dithiodiethanol at 35.2°, Danehy and Hunter (1967) obtained thiol (2-mercaptoethanol) in up to 80% yield, and a molar ratio of thiol to sulfonic acid (the only other product identified) of 4:1. They have concluded

that direct nucleophilic attack on sulfur is a possible mode of decomposition of 2,2′-dithiodiethanol, and that, in general, direct nucleophilic attack of hydroxide ion on sulfur initiates decomposition unless this is inhibited by close proximity of a negative charge. Schöberl and Tausent (1955) obtained an 80% yield of mercaptan upon alkaline hydrolysis of carboxymethyl β-carboxyethyldisulfide at 40°. Danehy (1966) suggests that the first two steps in the alkaline decomposition of dithiodiethanol and of dithiodipropionic acid are the same. In proteins, the attachment of the reactive functional groups to the polypeptide chain may limit both the rate and course of reactions leading to the final products of the hydrolysis of the disulfide bond by hydroxide ion.

Hydrolysis of the disulfide bond in aromatic disulfides appears to proceed by an obligatory S–S split (see Danehy and Hunter, 1967, for references). The $3RS^-$:2S–S product to reactant ratio suggested by the spectra of Figure 5 appears to confirm the hydrolysis as an S–S split. The lower activation energy observed here for alkaline hydrolysis of the disulfide bond of Ellman's reagent may be a result of the proximity of the aromatic ring to the disulfide bond.

The dependence of the rate constant for hydrolysis of the disulfide bonds of ovomucoid upon ionic strength (Figure 2) is qualitatively that expected for reaction of a negative ion with a protein of net negative charge. Such primary salt effects upon rate constants for reactions of proteins have been observed, for example, by Steinhardt (1937) for denaturation of pepsin, and by Cann (1964) for binding of zinc ion to myoglobin.

Alkaline hydrolysis of the disulfide bond produces conformational effects on ovomucoid. The large reduced viscosity produced at high pH, previously observed by Jirgensons *et al.* (1960), indicates considerable unfolding of ovomucoid in alkali. The effect on the optical rotation in the visible region is not great, and is probably due to two changes which are compensatory—an increase in rotation due to hydrolysis of disulfide bonds and a decrease in rotation due to denaturation (exposure of peptide bonds to solvent). Ikeda *et al.* (1968) observed a decrease in the magnitude of the circular dichroism bands of ovomucoid after 2 hr in 0.1 N NaOH, and interpreted the optical rotation changes observed near 230 mμ as showing a decrease in helical content. Heating ovomucoid, either in native form or after treatment with alkali (Figure 6A) produced only monotonic change in rotation with increase in temperature. The direction of the change in rotation is the reverse of that usually observed when a protein undergoes denaturation.

Although the evidence summarized above suggests that disulfide bonds in proteins and aliphatic compounds undergo S–S fission, both Cecil and McPhee (1959) and Tarbell and Harnish (1951) suggest that the weight of other experimental evidence favors β elimination for aliphatic disulfides, leading to an unsaturated product, IIA (a dehydroalanine residue when a protein undergoes hydrolysis at the disulfide bond), and a persulfide, IIB. Probably the best experimental evidence for the C–S split of mechanism II is that provided by Gawron and Odstrchel (1967), in their study of the alkaline hydrolysis of cystine endopeptides. However, the absorption spectrum of dehydroalanine (IIA) as given by Carter and Greenstein (1946) and reproduced in Figure 4 does not agree as well with the changes in absorption observed here as does the absorption spectrum of thiol anion. Also, Rao and Gorin (1959) state that the persulfide ion (RSS^-, IIB) absorbs at 335 mμ ($\epsilon_{mol} \simeq 310$). No absorption was observed near 335 mμ in the experiments reported here. Rosenthal and Oster (1961)

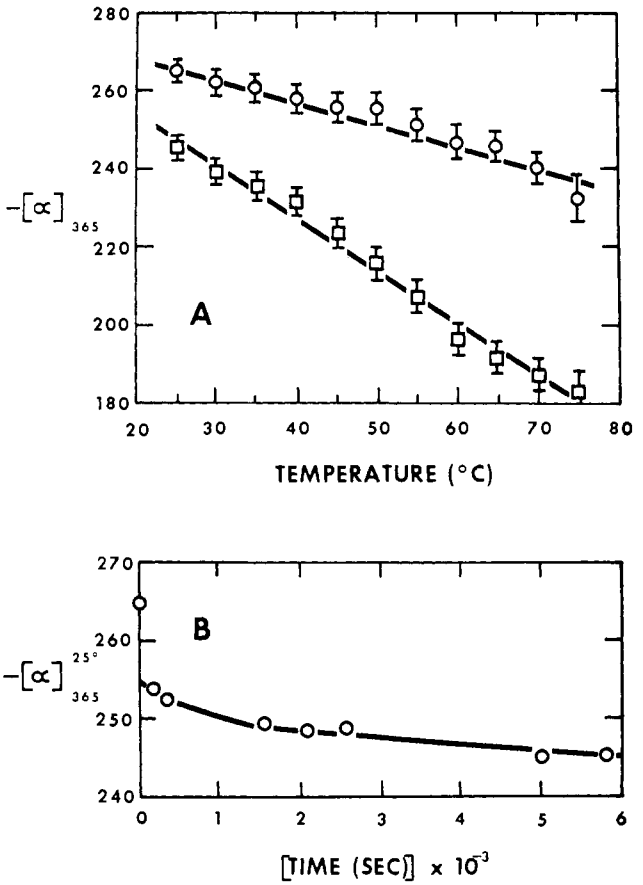


FIGURE 6: Effect of exposure to alkali on optical rotation of ovomucoid. (A) Change in rotation at 365 mμ with temperature at pH 7, before addition of alkali (○), at pH 12.8 after exposure to alkali at pH 12.8 (□). (B) Change in optical rotation with time at 25° when a neutral solution of ovomucoid is adjusted to pH 12.8.

suggest attack by hydroxide ion on the carbon atom α to the disulfide bond leads to a modified β elimination in which an S–S split occurs, but Swan (1957) considers this mechanism inadequate.

Recently, Andersson and Berg (1969) have demonstrated that in the presence of *p*-hydroxymercuribenzoate, oxidized glutathione is quantitatively converted by alkali into glutathione-*p*-hydroxymercuribenzoate mercaptide and glutathionesulfinic acid. The stoichiometry was observed to be that of eq 4. The reaction rate is first order with respect to time, independent of *p*-hydroxymercuribenzoate concentration, and directly proportional to hydroxide ion concentration. No splitting of carbon-to-sulfur bonds was observed below pH 11. Alkaline fission of the disulfide bond joining the monomers in bovine serum albumin dimer (Andersson, 1970) likewise resulted in no cleavage of carbon-to-sulfur bonds, as indicated by absence of lysinoalanine, but the reaction appeared to proceed largely by a series of disulfide–sulfhydryl-interchange reactions.

In the present experiments, the good agreement obtained between rate of loss of cystine and rate of appearance of thiol actually presents a problem. If the classical mechanism (I) is operative for proteins, then three-fourths of the sulfur should appear as product thiol, and be recovered as half-cystine after HCl hydrolysis and air oxidation to cystine before amino acid chromatography. The data of Figure 1 indicate that at longer times of exposure of ovomucoid to

alkali *no* half-cystine would be observed on amino acid analysis. The appearance of lysinoalanine presents another problem. Since the content of the amino acids serine and threonine was unchanged by alkali treatment (Figure 1), lysinoalanine could probably only have been produced from cystine or cysteine. It may have been formed by dehydration of product cysteine if S–S cleavage took place, as postulated. Although cysteine is more stable than cystine to alkaline treatment, it nevertheless is decomposed by alkali both by β elimination (C–S split) and by loss of ammonia (Andrews, 1928; Clarke and Inouye, 1930, 1932; Rivett *et al.*, 1965). This might account both for absence of the half-cystine expected at infinite time on the basis of mechanism I, and for the formation of lysinoalanine.

In summary, the main findings of the present experiments are as follows. (1) The activation energy for alkaline hydrolysis of disulfide bonds in ovomucoid and selected aliphatic disulfides, in the pH range 11–13, is 19 kcal/mole. (2) Increase in ionic strength increases the rate of hydrolysis of the disulfide bonds of ovomucoid, but does not change the activation energy. (3) The rate of hydrolysis of the disulfide bonds of ovomucoid is 100- to 1000-fold faster than that of the aliphatic disulfides studied, but slower than that of an aromatic disulfide. (4) Thiol is produced in the rate-limiting step. (5) Production of thiol occurs at the same rate as loss of cystine. (6) There is quantitative agreement between both the rate and the amount of thiol produced (as measured with Ellman's reagent) and the rate and the amount of change in absorption at 240 mμ (average molar absorption coefficient of thiol taken as 5×10^3). (7) The experimental observations are consistent with S–S cleavage of the disulfide bond by hydroxide ion.

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