McGhee, J. D., von Hippel, P. H., *Biochemistry* **14**, 1281 (1975a).
McGhee, J. D., von Hippel, P. H., *Biochemistry* **14**, 1297 (1975b).
Means, G. E., Feeney, R. E., *Biochemistry* **7**, 2192 (1968).
Means, G. E., Feeney, R. E., "Chemical Modification of Proteins", Holden-Day, San Francisco, Calif., 1971.
Mitchell, J., Jr., *Org. Anal.* **1**, 243 (1953).
Mohammadzadeh-K., A., Smith, L. M., Feeney, R. E., *Biochim. Biophys. Acta* **194**, 256 (1969).
Myers, J. S., Hardman, J. K., *J. Biol. Chem.* **246**, 3863 (1971).
Ottesen, M., Svensson, B., *Lab. Carlsberg* **38**, 445 (1971).
Reis, P. J., Tunks, D. A., *Aust. J. Biol. Sci.* **26**, 1127 (1973).
Rice, R. H., Means, G. E., *J. Biol. Chem.* **246**, 831 (1971).
Scott, T. W., Cook, L. J., Mills, S. C., *J. Am. Oil Chem. Soc.* **48**, 358 (1971).
Warren, J. R., Spero, L., Metzger, J. F., *Biochim. Biophys. Acta* **365**, 434 (1974).
Zittle, C. A., *J. Franklin Inst.* **246**, 266 (1948).

Received for review July 12, 1976. Accepted November 1, 1976. This research was supported in part by Grant No. AM-13686 from the National Institutes of Health. F. Galembeck was supported by a postdoctoral fellowship from FAPESP (Brazil). Parts of this material were taken from the thesis of D. S. Ryan, submitted in partial fulfillment of the requirements for the Ph.D. in Biochemistry.

Effects of Alkali on Proteins. Disulfides and Their Products

Aws S. Nashef, David T. Osuga, Honson S. Lee, Ahmed I. Ahmed,[1] John R. Whitaker, and Robert E. Feeney*

Alkali treatment of disulfide-containing proteins with different structures and properties resulted in the formation of similar type products, but with different energies of activation. The principal protein studied was lysozyme, with comparative studies on bovine pancreatic ribonuclease, bovine α-lactalbumin, bovine serum albumin, chicken ovotransferrin, and several avian ovomucoids. Alkali treatment of proteins (10^{-5} M protein in 0.1 M NaOH at 50 °C for 24 h) resulted in the loss of cystine and lysine and the formation of new amino acids. Alkali treatment was accompanied by an increase in absorbance at 241 nm with time until it reached a maximum at which time it started decreasing and finally plateaued. The rate of increase in absorbance at 241 nm was found to be a function of both base and disulfide concentration. The mechanism of action appeared to involve a β elimination of the disulfides resulting in the intermediate, dehydroalanine. Michael-type nucleophilic additions of the ϵ-amino groups of lysine, the sulfur of cysteine, and the nitrogen of ammonia to the double bond of the dehydroalanine lead to the formation of lysinoalanine, lanthionine, and β-aminoalanine, respectively. The energy of activation (E_a) for several disulfide-containing proteins was in the range of 14.2 kcal/mol for Golden-Amherst pheasant cross ovomucoid to a high of 23.8 kcal/mol for lysozyme, while the change in free energy, ΔF^*, was essentially the same (20.2 ± 0.2 kcal/mol) for all proteins.

Alkali treatment of proteins is increasingly employed for obtaining products with certain desirable technological characteristics such as protein concentrates and isolates, foaming, emulsifying and stabilizing agents, and spun fibers (DeGroot and Slump, 1969; Meyer and Williams, 1977). Alkali is listed under the GRAS (generally recognized as safe) list. Nevertheless, it has been conclusively shown that alkali-treated proteins show racemization, losses in some amino acids, and the formation of unnatural products (Spande et al., 1970; Woodard et al., 1975; Sternberg et al., 1975; Gross, 1977; Feeney, 1977).

Although the alkaline decomposition of various disulfides has been studied extensively (Schöberl and Wagner, 1955, 1958; Parker and Kharasch, 1959; Cecil and McPhee, 1959; Foss, 1961; Gawron, 1966; Danehy, 1966, 1971; Spande et al., 1970; Friedman, 1973), there is still considerable controversy over the mechanism of action of aqueous alkali on disulfides. Three mechanisms have been proposed to explain the various products obtained when different disulfide compounds were treated with aqueous alkali: (1) hydrolysis or nucleophilic substitution which involves displacement of sulfur from sulfur by hydroxide ion (Schiller and Otto, 1876; Schöberl, 1933, 1937; Schöberl et al., 1934; Schöberl and Eck, 1936); (2) α elimination which involves the initial abstraction of proton on a carbon α to a sulfur followed by heterolytic cleavage of the sulfur–sulfur bond giving a mercaptide anion and thioaldehyde or thioketone which would decompose further into an aldehyde or ketone and hydrogen sulfide (Rosenthal and Oster, 1954); and (3) β elimination which involves abstraction of the acidic proton from a carbon β to a sulfur atom, followed by the rearrangement of the carbanion formed into an olefin and an unstable disulfide anion (persulfide) which would further decompose to give a mercaptide anion and elemental sulfur (Bergmann and Stather, 1926; Nicolet, 1931; Tarbell and Harnish, 1951).

The aim of this work has been to elucidate the mechanism of action of hydroxide ion on disulfide bonds in proteins, as well as to describe some of the kinetic parameters for the overall reaction and some of the products.

MATERIALS AND METHODS

Ribonuclease A, lot 107B-8001, bovine serum albumin, lot 23C-8150, bovine pancreatic insulin, lot 24C-3130, and purified bovine α-lactalbumin, Grade II, lot 15C-7080, were purchased from Sigma Chemical Company. Cell walls of *Micrococcus lysodeikticus*, lot C606-2, were from Worthington Biochemical Corporation; L(+)-*meso*-lan-

Department of Food Science and Technology, University of California, Davis, California 95616.
[1]Present address: Biochemistry Department, Cairo University, Giza, Egypt.

thionine and DL-lanthionine were from Pfaltz and Bauer Inc.; β-aminoalanine monohydrobromide, lot 030167, was from Aldrich; and lysinoalanine was a gift from Dr. John W. Finley, Western Regional Research Laboratory, ARS, USDA, Berkeley, Calif. Chicken, turkey, penguin, and Golden-Amherst pheasant cross (GAX) ovomucoids (Osuga et al., 1974) and iron-free chicken ovotransferrin (Greene and Feeney, 1968) were pure fractions isolated in our laboratory. All other reagents were of analytical grade. Deionized water was used.

Isolation and Purification of Lysozyme. Lysozyme crystals were isolated from chicken egg white according to the method of Alderton and Fevold (1946). Lysozyme was further purified by ion-exchange chromatography and gel filtration, according to the method described by Arnheim et al. (1969). The dialyzed lysozyme solution was not lyophilized but rather kept frozen because lyophilization caused association as was indicated by gel electrophoresis. The lysozyme was found to be pure by disc and sodium dodecyl sulfate gel electrophoresis and by activity measurements.

Alkali Treatment and Spectral Analysis. Alkaline solutions at different pH values were prepared according to Bates (1973). Other alkaline solutions were various concentrations of sodium hydroxide in water. Protein stock solutions were made 3×10^{-4} M in either 0.05 M KCl or 0.025 M $NaHCO_3$, depending on the pH of the solution to be used. A volume of 2.9 ml of the desired base solution was pipetted into a cuvette which was incubated in a thermostated cuvette holder in a Cary 118-C spectrophotometer until the temperature was equilibrated (usually 10 min). Then, 0.1 ml of the stock protein solution was added to provide a final concentration of 1×10^{-5} M. The contents were mixed and A_{241} was recorded with time. A spectrum of the alkali-treated protein was also recorded with time. To test for possible effects of oxygen, spectra were obtained in the presence and absence of oxygen.

Determination of Thiol Produced. The amount of thiol was quantitated with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) according to the procedure of Ellman (1959) with slight modification. Alkali treatment was stopped at different time intervals with concentrated phosphoric acid to give a final pH of 8. Twenty-five microliters of Nbs_2 solution (39.6 mg of Nbs_2 in 10 ml of 0.1 M phosphate buffer, pH 7.0) was added, and A_{412} of the solution was determined after 5 min. The concentration of thiol produced was calculated using the molar extinction coefficient of 13 600 M^{-1} cm^{-1}.

Amino Acid Analysis. For alkali treatment, a 1% solution of the protein in 0.1 M NaOH was incubated in Pyrex test tubes at 50 °C for different periods of time. The reaction was stopped with an equal volume of concentrated HCl. The tubes were evacuated, sealed, and kept at 110 °C for 22 h. HCl was evaporated and the samples were dissolved in 1×10^{-3} N HCl or pH 2.71 buffer, initial buffer for amino acid analysis. Quantitation of amino acids lost and new ones formed was done on a Technicon amino acid analyzer. A modification of the standard Technicon technique was used to elute all amino acids (pH 2.71 was used instead of pH 2.87 as initial buffer), while pH 5.1 buffer was used for basic amino acid analysis.

Determination of Lysozyme Activity. Lysozyme activity was followed using the lysis method described by Fraenkel-Conrat (1950). Specific activity was defined as ΔA_{540} min^{-1} mg^{-1} of lysozyme.

Quantitation of Hydrogen Sulfide and Elemental Sulfur. Quantitations of both free elemental sulfur and hydrogen sulfide were done according to Johnson and Nishita (1952). Duplicate samples of 2×10^{-5} M lysozyme incubated at various pH values at 50 °C for 24 h were analyzed and compared with other duplicate samples treated with acid followed by bubbling nitrogen through the acidified samples to remove H_2S. By this method, elemental sulfur and H_2S are then calculated from the differences between total free sulfur and the sulfur removed after acidification.

H_2S was also determined by trapping it from the nitrogen used for purging (bubbling) of the acidified samples and titration of the H_2S with Nbs_2 as described elsewhere (Nashef et al., 1977).

RESULTS

Spectra of Alkali-Treated Proteins. Three things were apparent by comparing the spectra of native and alkali-treated proteins (Figure 1): (1) an increase in absorption in the aromatic region, (2) a shift of the absorption maxima to longer wavelength, and (3) an increase in absorption at 241 nm with time. The first two have been ascribed to the ionization of tyrosine (Shugar, 1952). The absorption at 241 nm may be due to the formation of sulfide (Donovan and White, 1971) and dehydro amino acids (Bohak, 1964). In the present study it was used as an approximate method to follow the reaction rates.

Effects of Conditions on Alkali-Mediated Changes in A_{241}. Alkali treatment caused an increase in A_{241} with time (Figure 1). The maximum increase was also proportional to both base concentration (Figure 2) and disulfide concentration (Figure 3).

Alkali treatment of lysozyme at pH values of 12 and lower at 50 °C resulted in turbidity which interfered with the spectral analysis but not with the Nbs_2 analysis (see below). Ovotransferrin, ovomucoid, and ribonuclease did not show this effect. Treatment of lysozyme in alkaline solution made 8 M in urea caused an increase in initial rates and in absorption at 241 nm (Figure 4). No turbidity was noticed when urea was present.

Table I shows the energies of activation calculated from the initial rates of reaction of hydroxide ion with disulfides in different proteins: lysozyme, ribonuclease, insulin, bovine serum albumin, and the ovomucoids from the chicken, turkey, penguin, and GAX.

Products of Alkali-Treated Proteins. The formation of thiol could also be used to monitor the alkali-mediated changes (Figure 5). Thiol production increased with time, but this did not correspond to the observed increase in A_{241}. Nbs_2 and spectral analysis of lysozyme, ribonuclease, and the different ovomucoids indicated that the initial rate of thiol produced contributed about one-half the observed initial rate of increase in A_{241}.

The amino acids found to be affected by alkali incubation were cystine and lysine. New amino acids, lanthionine, lysinoalanine, and β-aminoalanine, were identified and quantitated. Table II shows the losses of some amino acids as well as the formation of new products with time of alkali treatment. Figure 6 shows the elution pattern of *meso*- and DL-lanthionine, and Figure 7 the elution pattern of lysinoalanine and β-aminoalanine. Lysinoalanine was confirmed using periodate treatment which resulted in the absence of the peak for lysinoalanine and increase in the lysine peak. Sodium dodecyl sulfate gel electrophoresis of the alkali-treated lysozyme indicated intermolecular cross-linking as well as the hydrolysis of one bond resulting in a small peptide.

When the 1440-min sample from Table II was examined for free sulfur, it was found that the unacidified samples of alkali-treated lysozyme contained a total of 6.55 mol of total free sulfur per mol of lysozyme, while the acidified

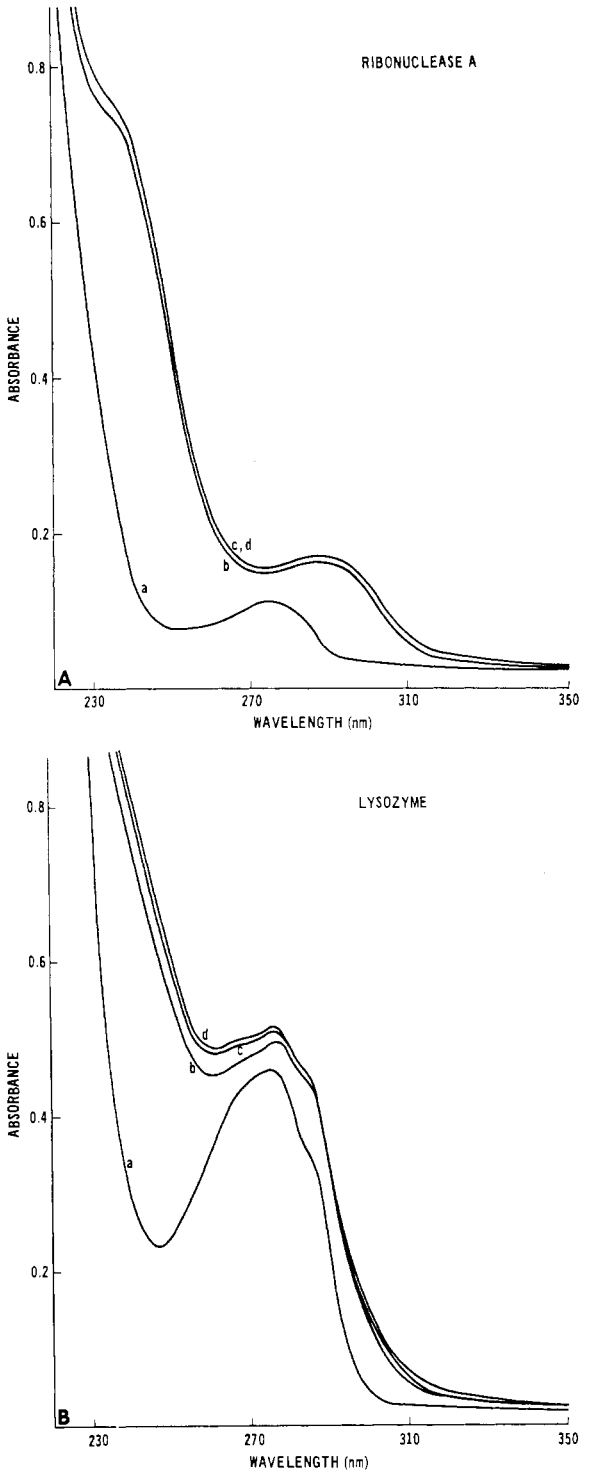


Figure 1. Absorption spectra for (A) ribonuclease A (1×10^{-5} M) and (B) lysozyme (1.2×10^{-5} M) at 50 °C in the following solutions: (a) H_2O, (b, c, and d) 0.1 M NaOH for 1, 3, and 6 min, respectively.

and N_2 purged ones contained a total of 3.75 mol of sulfur per mol of lysozyme. This suggested that H_2S amounted to 2.8 mol (~3) per mol of lysozyme while elemental sulfur amounted to 3.75 mol (~4) per mol of lysozyme.

The formation and quantitation of H_2S were confirmed by absorption of the H_2S into alkaline solution from the N_2 used to purge the acidified solutions (Nashef et al., 1977). In agreement with the indirect method, 2.8 to 3.1 mol of H_2S per mol of lysozyme were found.

Effects of Alkali Treatment on Enzymatic Activity of Lysozyme. Lysozyme is rapidly inactivated in weakly alkaline (pH 9) solutions in the presence of cupric ion but is relatively stable up to pH 10 in the absence of metal ions (Feeney et al., 1956). In the present studies, losses of activities paralleled A_{241} changes. Figure 8 shows the specific activity as a function of time of alkali treatment of 1×10^{-5} M lysozyme in (a) 0.5 M NaOH at room temperature and (b) in 0.1 M NaOH at 50 °C.

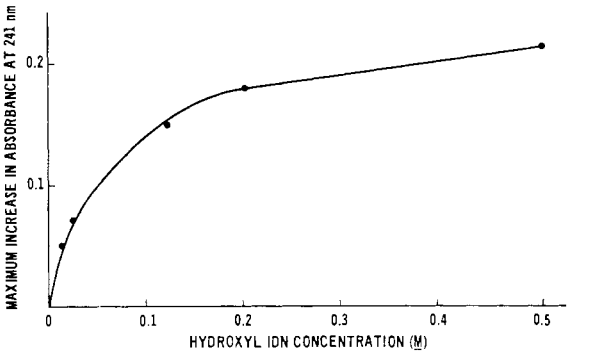


Figure 2. Maximum increase in absorbance at 241 nm as a function of hydroxyl ion concentration (M) used for the alkali treatment of 1×10^{-5} M lysozyme at 50 °C for approximately 6 min.

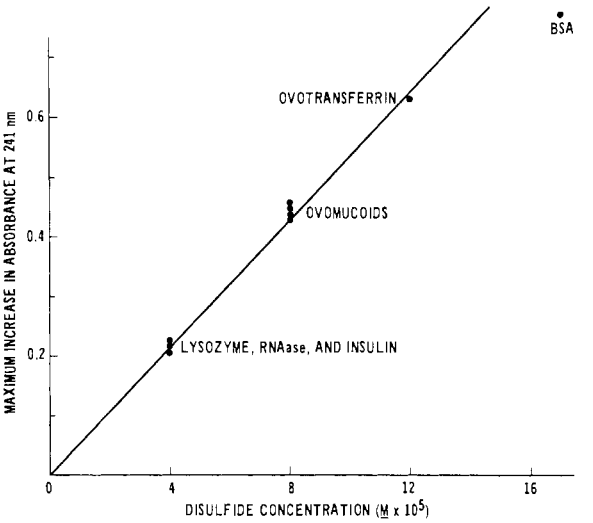


Figure 3. Maximal increase in A_{241} after alkali treatment as a function of concentration of disulfides in different proteins: lysozyme, ribonuclease A (RNase), and insulin; chicken, turkey, penguin, and Golden-Amherst pheasant cross (GAX) ovomucoids; ovotransferrin and bovine serum albumin (BSA).

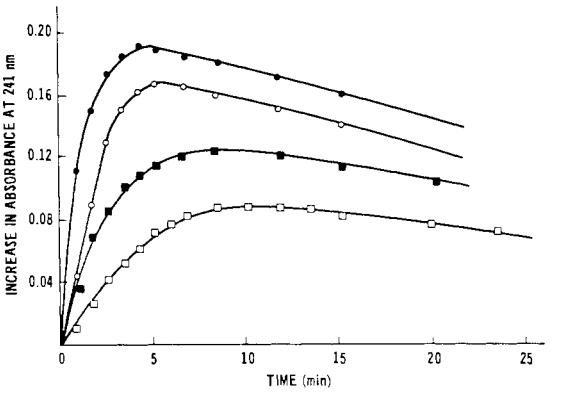


Figure 4. Increase in absorbance at 241 nm as a function of time of alkali treatment of 1×10^{-5} M lysozyme at 50 °C: (□) pH 12.4 solution; (■) pH 12.4 solution made 8 M in urea; (○) pH 13.2 solution; and (●) pH 13.2 solution made 8 M in urea.

Table I. Thermodynamic Activation Parameters for the β-Elimination Mechanism in Several Disulfide-Containing Proteins[a]

Protein[b]	Disulfide content, mol of disulfide/ mol of protein	E_a, kcal/mol	$\Delta H^{\ddagger}$, kcal/mol	$\Delta F^{\ddagger}$,[g] kcal/mol	$\Delta S^{\ddagger}$,[g] kcal/mol
Lysozyme	4[c]	23.8	23.1	20.2	8.71
Insulin	4[d]	22.9	22.2	20.5	5.11
Ribonuclease A	4[e]	19.9	19.2	20.0	−2.40
Bovine serum albumin	17[f]	19.9	19.2	20.3	−3.30
Chicken ovomucoid	8[c]	18.1	17.4	20.0	−7.81
Turkey ovomucoid	8[c]	18.1	17.4	20.1	−8.11
Penguin ovomucoid	8[c]	15.7	15.0	20.2	−15.6
GAX ovomucoid	8[c]	14.2	13.5	20.3	−20.4

[a] Initial rates were calculated by first dividing the initial change in OD_{241} s^{-1} M^{-1} of protein by disulfide content, followed by converting to dehydroalanine equivalent using $E_{241} = 4.2 \times 10^3$ M^{-1} cm^{-1} (Carter and Greenstein, 1946). The value obtained is then divided by hydroxide ion concentration (0.1 M) since $\kappa = \kappa'/[OH]$ (pseudo-first-order kinetics). [b] Protein concentrations were 1×10^{-5} M in 0.1 M NaOH at different temperatures. [c] Taken from Feeney and Allison (1969). [d] Taken from Sanger (1955). [e] Taken from Patchornik and Sokolovsky (1964). [f] Taken from Brown (1976). [g] Calculated from data obtained at 60 °C.

Table II. The Rates of Amino Acids Lost and Formed in Alkali-Treated Lysozyme[a]

Time, min	Amino acids lost, mol/mol		Amino acids formed, mol/mol		
	Cystine[b]	Lysine[c]	Lanthionine[d]	Lysinoalanine	β-Aminoalanine
1	0.56	0	0	0	0
3	1.50	0.31	0.19	0.27	0
6	2.32	0.46	0.56	0.43	0
15	3.16	1.35	0.68	1.41	0
30	3.76	2.15	0.86	2.13	0
60	4.0	2.52	1.05	2.68	0
1440	4.0	5.04	1.06	5.11	1.08

[a] Lysozyme 2×10^{-4} M in 0.1 M NaOH at 50 °C. [b] Total of four in lysozyme. [c] Total of six in lysozyme. [d] Sum of both meso and DL forms.

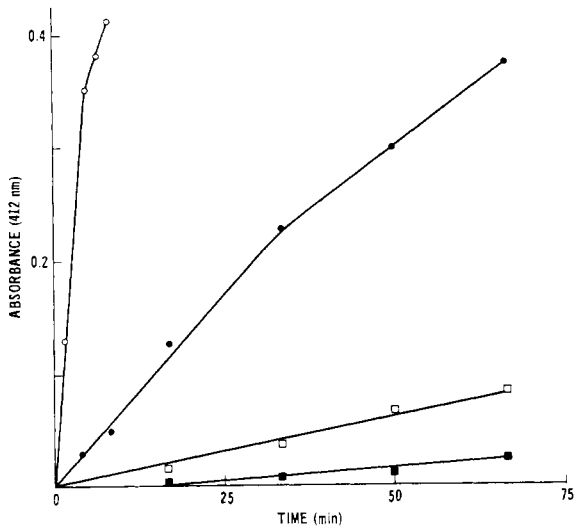


Figure 5. Absorbance at 412 nm as a function of time of alkali treatment of 1×10^{-5} M lysozyme at 50 °C and pH: (■) pH 10; (□) pH 11; (●) pH 12; and (○) pH 13. Amount of thiol produced was quantitated using 5,5′-dithiobis(2-nitrobenzoic acid).

Alkali Treatment of Aromatic Disulfides (Nbs_2). The change in A_{412} was in agreement with the stoichiometry of three thiols produced per two disulfides.

DISCUSSION

We have interpreted the results of the present study as supporting β elimination as the main, if not only, mechanism for the alkali-mediated covalent changes in proteins. The β-elimination mechanism can be represented by eq 1–4.

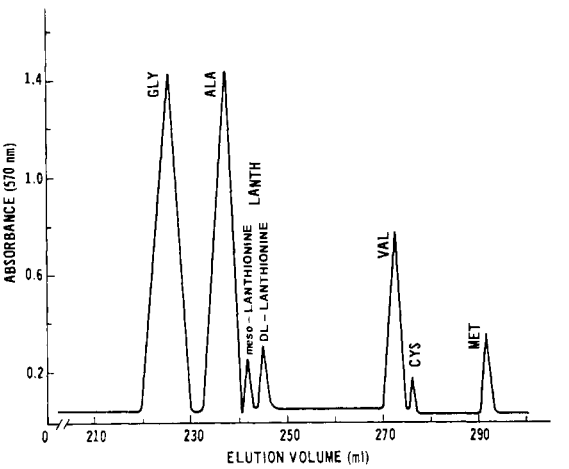


Figure 6. Absorbance at 570 nm as a function of elution volume (milliliters) of amino acids of a hydrolysate in alkali-treated lysozyme (1×10^{-5} M in 0.1 M NaOH, at 50 °C), showing the position of the new amino acids, *meso*- and DL-lanthionine (LANTH). The run was done on a Technicon amino acid analyzer, 21-h run as specified in Technicon manuals, but using pH 2.71–5.0 gradient for the buffer.

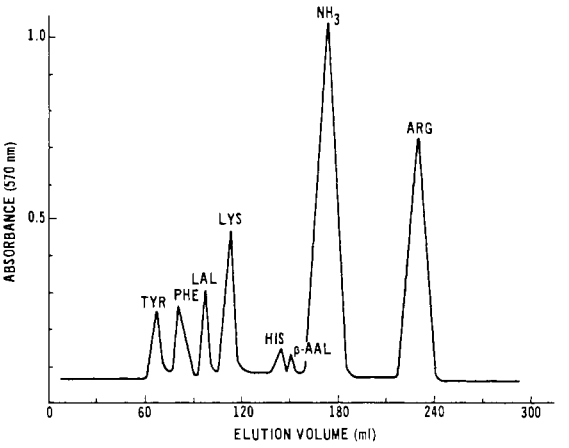


Figure 7. Absorbance at 570 nm as a function of elution volume (milliliters) of the basic amino acids of a hydrolysate of alkali-treated lysozyme (1×10^{-5} M, 0.1 M NaOH at 50 °C). The positions of the new amino acids, lysinoalanine (LAL) and β-aminoalanine (β-AAL), are shown. The run was done on a Technicon amino acid analyzer using pH 5.1 buffer for the elution of the basic amino acids.

Initially, the base abstracts the acidic α hydrogen of the cystine residue producing a carbanion (eq 1) which rearranges to give a dehydroalanyl residue and a persulfide

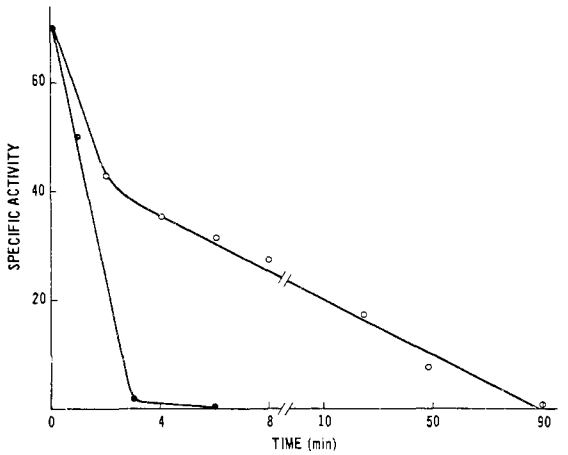
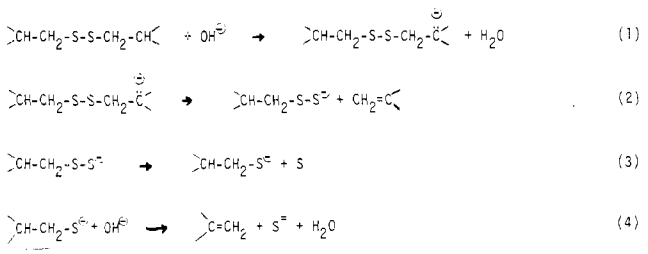
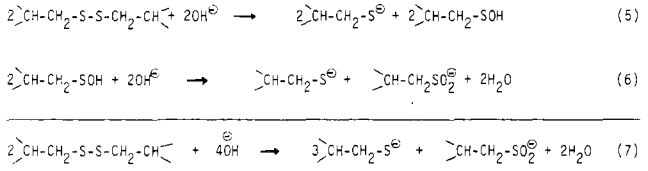


Figure 8. Specific activity of lysozyme as a function of time of alkali treatment in (○) 0.5 M NaOH at room temperature and (●) 0.1 M NaOH at 50 °C.



(thiocysteinyl residue) (eq 2) which is unstable and decomposes further to cysteinyl residue and elemental sulfur (eq 3). Cysteinyl residues may also undergo β elimination giving dehydroalanyl residues and sulfide as sodium sulfide (eq 4).

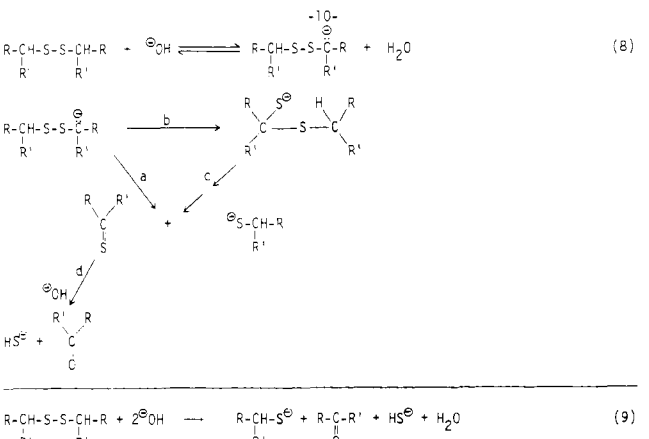
The hydrolysis mechanism can be represented by eq 5–7



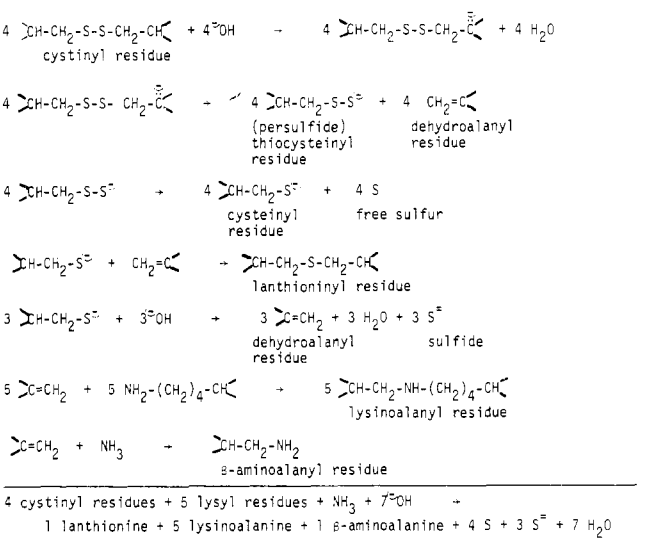
(Schiller and Otto, 1876). This involves the initial substitution of sulfur from another sulfur with hydroxyl giving a mercaptide anion and a sulfenic acid (eq 5) which would further disproportionate into a mercaptide anion and a sulfinic acid (eq 6). The overall stoichiometry is shown in eq 7.

The α-elimination mechanism, first proposed by Rosenthal and Oster (1954), may be illustrated by Scheme

Scheme I



Scheme II



I (Danehy and Elia, 1971). Initially the base abstracts hydrogen from a carbon α to a sulfur resulting in a carbanion (eq 8) which may rearrange either via route a or b,c to give a mercaptide and thioketone or thioaldehyde, depending on R′, which would proceed via route d to give hydrogen sulfide and the corresponding ketone or aldehyde. The overall stoichiometry is shown in eq 9.

The results of amino acid analysis of alkali-treated lysozyme and α-lactalbumin (2×10^{-4} M in 0.1 M NaOH at 50 °C) have shown that for each mole of protein, 1 mol of lanthionine (half *meso*- and half DL-), 5 mol of lysinoalanine, and 1 mol of β-aminoalanine are formed at the end of 24 h. This was accompanied by the complete loss of all four cystines and five lysines. Asquith et al. (1969) have reported the formation of lysinoalanine and β-aminoalanine upon alkali treatment of lysozyme, but did not report formation of lanthionine. Sulfur and hydrogen sulfide analyses of alkali-treated lysozyme (2×10^{-4} M in 0.1 M NaOH at 50 °C) indicated that for each mole of protein, 3 mol of hydrogen sulfide and 4 mol of sulfur are formed after 24 h. The presence of free sulfur, which has long been known to be formed (Mulder, 1838), cannot be accounted for by either the hydrolysis or α-elimination mechanisms. Although hydrogen sulfide can form from β elimination of the mercaptide anion formed in both hydrolysis and α-elimination mechanisms, it still does not account for the results. Our results fit the β-elimination scheme (Scheme II). The formation of meso and DL forms of lanthionine provides more evidence in support of a carbanion intermediate in a Michael addition type reaction where hydrogen can add back from either side of the plane. Our data suggest that the formation of the new amino acids depends greatly on the presence of the right nucleophile in the vicinity of the double bond of dehydroalanyl residues rather than being due to differences in rates. Although it seems that lysinoalanine formation is favored, this could probably be due to the long side chain of lysyl residues. Alkali treatment of Nbs_2 on the other hand seems to proceed via the hydrolysis mechanism, where β elimination and α elimination are not possible. This was also established by Donovan (1967) and Danehy et al. (1971). Danehy and Parameswaran (1968) have shown that the alkaline decomposition of ten aromatic disulfides follows the generalization of Parker and Kharasch (1960) that the disulfide which gives rise to a more stable anion should decompose more rapidly than the one that gives a less stable anion. Although evidence in support of the β-

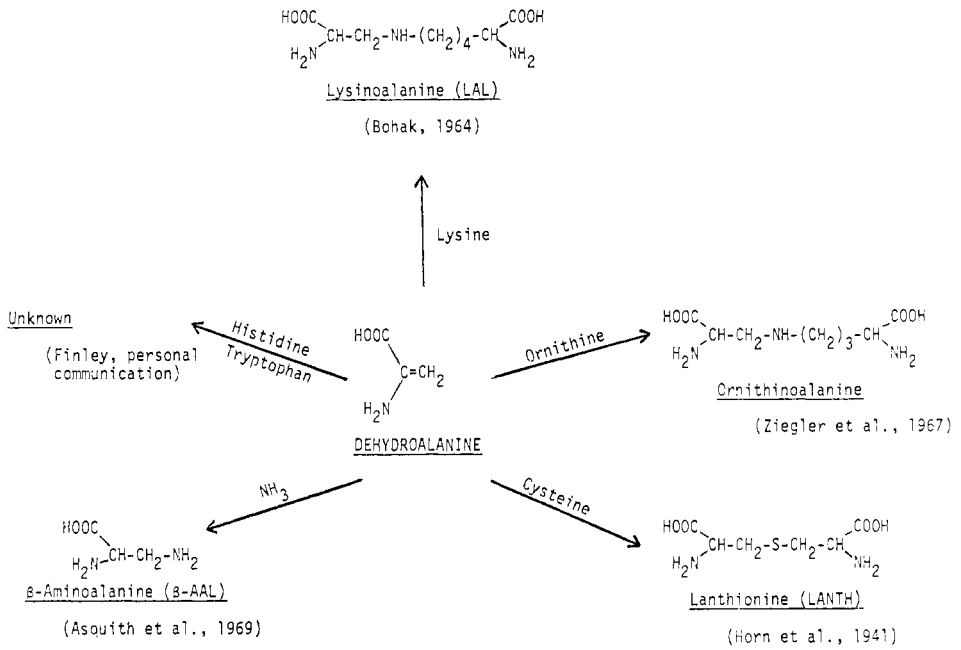


Figure 9. Possible products that may form through nucleophilic addition (Michael-type addition) to a dehydroalanine intermediate in alkali-treated proteins.

elimination mechanism in proteins has been presented by Tarbell and Harnish (1951), Swan (1957), Patchornik and Sokolovsky (1964), Sokolovsky et al. (1964), Ziegler (1964), Bohak (1964), Corfield et al. (1967), Miro and Garcia-Dominguez (1967), and Gawron and Odstrschel (1967), the stoichiometry was never established. We have presented strong evidence by establishing the correct stoichiometry.

The increase in A_{241nm} was found to be a function of base concentration (Figure 2) and disulfide concentration (Figure 3). Unfolding of the protein using 8 M urea caused an increased rate of change in 241-nm absorption (Figure 4). Increasing ionic strength did not produce an appreciable increase in rate of change in A_{241nm} under the alkali treatment conditions employed (1×10^{-5} M lysozyme in 0.1 M NaOH at 50 °C). With proteins such as the ovomucoids and ribonuclease, which contain no, or very small amounts of tryptophan, the absorption maximum at approximately 275 nm shifted to higher wavelengths around 290 nm in alkali (Figure 1A), typical of tyrosine (Shugar, 1952), while this effect was less noticeable with proteins having higher ratios of tryptophan to tyrosine, such as lysozyme (Figure 1B).

Values for energies of activation of different proteins (Table I) were in the range of the values of 21 and 18.8 ± 0.5 kcal/mol reported for *N,N'*-dicarbobenzyloxy-L-cystinylglycine (Gawron and Odstrschel, 1967) and chicken ovomucoid (Donovan and White, 1971), respectively. It is interesting to note that penguin and GAX ovomucoid gave lower values. The change in free energy (ΔF^*) at 60 °C for the different proteins was essentially the same within experimental error for all proteins tested (Table I). Any changes in enthalpy (ΔH^*) were compensated by changes in entropy (ΔS^*). Donovan and White (1971) proposed that the mechanism is a hydrolysis mechanism, and suggested that the absorption at 241 nm may be due to thiol formation as determined by analysis with Nbs_2. It is noteworthy to mention that we have found that hydrogen sulfide, which is formed during the alkaline treatment of proteins, gives a stoichiometry of 2 mol of 5-thio-2-nitrobenzoate anions per mol of hydrogen sulfide (Nashef et al., 1977), which suggests that quantitation of the amount of thiol produced with Nbs_2, assuming a stoichiometry of 1:1, would lead to erroneous results. Also, Donovan and White (1971) did not report the formation of *meso*- and DL-lanthionine in chicken ovomucoid, which we found to occur upon alkali treatment.

The formation of new amino acids (Figure 9) in alkaline solution is possible not only in pure protein systems described in this investigation but also in protein food products. DeGroot and Slump (1969), among other workers, reported on the formation of lysinoalanine in high protein foods which increased with more severe conditions of pH, temperature, and time. Alkaline treatment may lead to changes in the physical and chemical properties of proteins as well as in their nutritive value. Toxicological and nutritional studies on rats fed alkali-treated soy protein showed renal lesions and generally reduced digestibility and nutritive value of the protein (DeGroot and Slump, 1969; Woodard and Short, 1973; Van Beek et al., 1974). Using semipurified diets, Woodard and Short (1975) found that lysinoalanine was responsible for the renal lesions. Recently, however, lysinoalanine has been found widely distributed in a variety of home cooked and commercial foods and ingredients (Sternberg et al., 1975). A more thorough understanding of the reactions involved in the chemical changes occurring on alkali treatment of proteins may make it possible not only to better prevent the changes, but also to control them to form less deleterious changes.

ACKNOWLEDGMENT

The authors thank James Quick for running the colorimetric determinations of sulfur and sulfides. Appreciation is due to Clara Robison and Gail Nilson for typing of the manuscript and Chris Howland for editorial assistance.

LITERATURE CITED

Alderton, G., Fevold, H. L., *J. Biol. Chem.* **164**, 1 (1946).
Arnheim, N., Prager, E. M., Wilson, A., *J. Biol. Chem.* **244**, 2085 (1969).
Asquith, R. S., Booth, A. K., Skinner, J. D., *Biochim. Biophys. Acta* 181, 164 (1969).
Bates, R. G., "Determination of pH, Theory and Practice", Wiley, New York, N.Y., 1973.

Bergmann, M., Stather, F., *Z. Physiol. Chem.* **152**, 189 (1926).
Bohak, Z., *J. Biol. Chem.* **239**, 2878 (1964).
Brown, J. R., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **35**, 1621 (no. 1335) (1976).
Carter, C. E., Greenstein, J. P., *J. Biol. Chem.*, **165**, 725 (1946).
Cecil, R., McPhee, J. R., *Adv. Protein Chem.* **14**, 256 (1959).
Corfield, M. C., Wood, C., Robson, A., Williams, M. J., Woodhouse, J. M., *Biochem. J.* **103**, 156 (1967).
Danehy, J. P., in "The Chemistry of Organic Sulfur Compounds", Vol. 2, Kharasch, N., Meyers, Y., Ed., Pergamon Press, New York, N.Y., 1966, p 337.
Danehy, J. P., *Int. J. Sulfur Chem., Part B* **6**(2), 103 (1971).
Danehy, J. P., Elia, V. J., *J. Org. Chem.* **36**, 1394 (1971).
Danehy, J. P., Elia, V. J., Lavelle, C. J., *J. Org. Chem.* **36**, 1003 (1971).
Danehy, J. P., Parameswaran, K. N., *J. Org. Chem.* **33**, 568 (1968).
DeGroot, A. P., Slump, P., *J. Nutr.* **98**, 45 (1969).
Donovan, J. W., *Biochem. Biophys. Res. Commun.* **29**, 734 (1967).
Donovan, J. W., White, T. M., *Biochemistry* **10**, 32 (1971).
Ellman, G. L., *Arch. Biochem. Biophys.* **82**, 70 (1959).
Feeney, R. E., *Adv. Chem. Ser.*, in press (1977).
Feeney, R. E., Allison, R. G., "Evolutionary Biochemistry of Proteins. Homologous and Analogous Proteins from Avian Egg Whites, Blood Sera, Milk, and Other Substances", Wiley, New York, N.Y., 1969.
Feeney, R. E., MacDonnell, L. R., Ducay, E. D., *Arch. Biochem. Biophys.* **61**, 72 (1956).
Finley, J. W., personal communication, 1975.
Foss, O., in "Organic Sulfur Compounds", Vol. 1, Kharasch, N., Ed., Pergamon Press, New York, N.Y., 1961, p 83.
Fraenkel-Conrat, H., *Arch. Biochem. Biophys.* **27**, 109 (1950).
Friedman, M., "The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins", Pergamon Press, New York, N.Y., 1973.
Gawron, O., in "The Chemistry of Organic Sulfur Compounds", Vol. 2, Kharasch, N., Meyers, Y., Ed., Pergamon Press, New York, N.Y., 1966, p 351.
Gawron, O., Odstrschel, G., *J. Am. Chem. Soc.* **89**, 3263 (1967).
Greene, F. C., Feeney, R. E., *Biochemistry* **7**, 1366 (1968).
Gross, E., *Adv. Chem. Ser.*, in press (1977).
Horn, M. J., Jones, D. B., Ringel, J. J., *J. Biol. Chem.* **138**, 141 (1941).
Johnson, C. M., Nishita, H., *Anal. Chem.* **24**, 736 (1952).
Meyer, E. W., Williams, L. D., *Adv. Chem. Ser.*, in press (1977).
Miro, P., Garcia-Dominguez, J., *J. Soc. Dyers Colour.* **83**, 91 (1967).
Mulder, G. J., *Ann.* **28**, 73 (1838).
Nashef, A. S., Osuga, D. T., Feeney, R. E., *Anal. Biochem.*, in press (1977).
Nicolet, B. H., *J. Am. Chem. Soc.* **53**, 3066 (1931).
Osuga, D. T., Bigler, J. C., Uy, R. L., Sjöberg, L., Feeney, R. E., *Comp. Biochem. Physiol.* **48B**, 519 (1974).
Parker, A. J., Kharasch, N., *Chem. Rev.* **59**, 583 (1959).
Parker, A. J., Kharasch, N., *J. Am. Chem. Soc.* **82**, 3071 (1960).
Patchornik, A., Sokolovsky, M., *J. Am. Chem. Soc.* **86**, 1206 (1964).
Rosenthal, N. A., Oster, G., *J. Soc. Cosmet. Chem.* **5**, 286 (1954).
Sanger, F., *Bull. Soc. Chim. Biol.* **37**, 23 (1955).
Schiller, R., Otto, R., *Chem. Ber.* **9**, 1637 (1876).
Schöberl, A., *Ann.* **507**, 111 (1933).
Schöberl, A., *Ber. Dtsch. Chem. Ges. B* **70**, 1186 (1937).
Schöberl, A., Berninger, E., Harren, F., *Ber. Dtsch. Chem. Ges. B* **67**, 1545 (1934).
Schöberl, A., Eck, H., *Ann.* **522**, 97 (1936).
Schöberl, A., Wagner, A., "Houben-Weyl's Methoden der Organischen Chemie", Vol. 9, Thieme, Stuttgart, 1955.
Schöberl, A., Wagner, A., "Houben-Weyl's Methoden der Oganischen Chemie", Vol. 11(2), Thieme, Stuttgart, 1958.
Shugar, D., *Biochem. J.* **52**, 142 (1952).
Sokolovsky, M., Sadeh, T., Patchornik, A., *J. Am. Chem. Soc.* **86**, 1212 (1964).
Spande, T. F., Witkop, B., Degani, Y., Patchornik, A., *Adv. Protein Chem.* **24**, 97 (1970).
Sternberg, M., Kim, C. Y., Schwende, F. J., *Science* **190**, 992 (1975).
Swan, J. M., *Nature (London)* **133**, 930 (1957).
Tarbell, D. S., Harnish, D. P., *Chem. Rev.* **49**, 1 (1951).
Van Beek, L., Feron, V. J., DeGroot, A. P., *J. Nutr.* **104**, 1630 (1974).
Woodard, J. C., Short, D. D., *J. Nutr.* **103**, 569 (1973).
Woodard, J. C., Short, D. D., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **24**, Abstr. No. 4022 (1975).
Woodard, J. C., Short, D. D., Alvarez, M. R., Reyniers, J., in "Protein Nutritional Quality of Foods and Feeds", Vol. 2, Friedman, M., Ed., Marcel Dekker, New York, N.Y., 1975, p 595.
Ziegler, K., *J. Biol. Chem.* **239**, 2713 (1964).
Ziegler, K., Melchert, I., Lürken, C., *Nature (London)* **214**, 404 (1967).

Received for review September 1, 1976. Accepted December 10, 1976. This work was supported in part by National Institutes of Health Grant No. AM 13686 and Food and Drug Administration Grant No. FD 00568. This material constitutes research to be submitted to the Graduate Division of the University of California at Davis in partial fulfillment of the requirements for a Ph.D. degree in Agricultural Chemistry for A.S.N.