The method based on creatine determination is perhaps preferable because of its simplicity and speed. However, the use of the solubility method in conjunction with the creatine method would be helpful in verifying the amount of animal protein and in ruling out the possibility of adulteration of mixtures with exogenous creatine. For example, the ratio between extractable nitrogen (mg/g) and creatine (mg/g) is fairly constant in different beef muscles as well as in mixtures, varying between 7 and 9. A value lower than 7 would indicate adulteration of blends with exogenous creatine, while a value higher than 9 would indicate extensive loss of weep or drip as a result of mishandling of the meat samples. These parameters also would appear suitable for analyzing mixtures containing meat proteins other than beef. However, further work is required to determine the amount and variation in the content of PC breakdown products in poultry and pork muscles, and other edible animal tissues, as well as the effect of heating and cooking on this parameter.

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LITERATURE CITED

Dikeman, M. E., Tuma, H. J., Beecher, G. R., *J. Food Sci.* **36**, 190 (1971).
Flint, F. O., Lewin, Y. A., *J. Food Technol.* **11**, 137 (1976).
Hargreaves, L. L., Jarvis, B., Wood, J. M., "The Serological Detection of Soya in Meat Products", The British Food Manufacturers' Industrial Research Association, U.K. Research Report No. 206, 1974.
Jewell, G. C., "The Identification and Estimation of Soya Proteins in Sausages Using Light Microscopy Techniques", The British Food Manufacturers' Industrial Research Association, U.K. Technical Circular No. 571, 1974.
Lee, V. B., Greaser, M. L., Rickansrud, D. A., Hagberg, E. C., Briskey, E. J., *J. Food Res.* **40**, 380 (1975).
Rangeley, W. R. O., Lawrie, R. A., *J. Food Technol.* **11**, 143 (1976).
Robson, R. M., Goll, D. E., Temple, M. J., *Anal. Biochem.* **24**, 339 (1968).
Shelef, L. A., Morton, L. R., *Food Technol.* **30**, 44 (1976).
Wong, T., *Anal. Biochem.* **40**, 18 (1971).
Zarkadas, C. G., *Can. J. Biochem.* **53**, 96 (1975).

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# Reaction of Proteins with Formaldehyde in the Presence and Absence of Sodium Borohydride

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Lysozyme and ribonuclease reacted at approximately equal rates with small amounts of $^{14}C$-labeled formaldehyde to give stable derivatives containing 1–2 equiv of label. After 6 h of incubation of lysozyme with formaldehyde at room temperature and pH 9, monomers, dimers, and trimers were identified, along with traces of higher polymers. The monomers retained nearly full enzymatic activity and contained slightly over 1 mol of irreversibly bound formaldehyde. In contrast, on reductive methylation of casein by formaldehyde and $NaBH_4$, no unreacted formaldehyde was detected by chromotropic acid in less than 4 s after addition of the aldehyde. Side reactions, which lead to polymers in the absence of the $NaBH_4$, have not been seen under the conditions of reductive methylation. Low rates of chymotryptic hydrolysis of reductively methylated casein and bovine serum albumin were not due to compounds formed from such side reactions, but were shown to be due to product inhibition by the peptides formed by hydrolysis of the proteins.

Formaldehyde (HCHO) has long been extensively used in large amounts in the tanning and related industries (Feeney et al., 1975). The pharmaceutical industry has also used it for nearly 50 years in the preparation of toxoids, which are injected into humans. Although the total usage of microbial toxoids produced with HCHO is extensive, toxoids are administered to any one human individual only in relatively small amounts. More recently, however, HCHO is being used for products fed animals that are eventually eaten by humans. The current investigations on use of HCHO treatment to encapsulate lipids inside protein shells for ruminant feeds (Scott et al., 1971; Hemsley et al., 1973; Reis and Tunks, 1973) raise the possibility that significant amounts of the reaction products between the protein and HCHO could find their way into humans via initial incorporation into ruminants. Sheep fed HCHO-treated casein had four- to sixfold higher serum levels of $N^{\epsilon}$-methyllysine than did sheep fed casein which had not been treated with HCHO (Hemsley et al., 1973).

In spite of these uses of HCHO in feeding animals for human consumption, there is apparently only meager information on the nature of the compounds produced on treating proteins with HCHO (Feeney et al., 1975). Grisolia et al. (1975) have also recently focused attention on the possible role of aldehydes on the toxic effects of alcohols. Several alcohol dehydrogenases and a retinol dehydrogenase were rapidly inactivated by low concen-

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trations of acetaldehyde or formaldehyde.

HCHO is a uniquely reactive carbonyl compound (Feeney et al., 1975). Its initial reaction with most proteins is the reversible formation of hydroxymethyl adducts and Schiff bases with the amino groups of lysines. Reactions with thiols are also rapid, and other groups such as imidazoles, peptide bonds, amides, guanidines, and tyrosines have also been reported to react (Means and Feeney, 1971; Myers and Hardman, 1971; Dunlop et al., 1973; Bizzini and Raynaud, 1974; Warren et al., 1974; Feeney et al., 1975; Martin et al., 1975). Nuclear magnetic resonance studies have shown that one nitrogen of the imidazole ring of histidine reacts with formaldehyde to form an *N*-hydroxymethyl derivative in alkaline solution (Dunlop et al., 1973). Under acidic conditions, both nitrogen positions can form *N*-hydroxymethyl derivatives. Hydroxymethyl adducts in the presence of formaldehyde have also been reported with both the exocyclic and endocyclic nitrogens of purines and pyrimidines in DNA (McGhee and von Hippel, 1975a,b). A cross-linked product, a methylene-bridged lysine–tyrosine compound, has been isolated from acid hydrolysates of formaldehyde-treated tetanus and diphtheria toxins (Means and Feeney, 1971). More recent studies have indicated that, at neutral pH (7.5), staphylococcal enterotoxin B is polymerized by cross-linking between lysine residues, but at more acidic pH (5.0), polymerization is by cross-linking between the lysine and tyrosine residues (Bizzini and Raynaud, 1974). Covalent cross-links between serine and either glutamine or asparagine have also been reported in formaldehyde-treated proteins (Means and Feeney, 1971; Myers and Hardman, 1971; Feeney et al., 1975).

Means and Feeney (1968) reported that reductive alkylation of proteins by formaldehyde and $NaBH_4$ gave only stable, non-cross-linked, alkylated amino groups. Under mildly alkaline conditions (pH 8–9), extensive dimethylation of amino groups was achieved. Side reactions were prevented by adding the reducing agent to the protein before adding the carbonyl compound. Digestibility of the product by trypsin was decreased, apparently due primarily to the resistance of $N^{\epsilon}$,$N^{\epsilon}$-dimethyllysine residues to hydrolysis, as well as to some substrate inhibition at high levels of substrate (Lin et al., 1969a,b). Ottesen and Svensson (1971) confirmed the relatively high specificity of reductive alkylation with formaldehyde and $NaBH_4$ (Means and Feeney, 1968). However, they did not place much importance on the necessity of adding the reducing agent before the formaldehyde, and reported nonspecific reactions at high levels (Ottesen and Svensson, 1971).

The present study was done as a consequence of results of studies initially directed at the use of extensive dimethylation of proteins to block amino groups for food uses. Preliminary studies indicated that extensively reductively methylated casein was not hydrolyzed as readily by $\alpha$-chymotrypsin as was the unmodified casein. This suggested that other groups in addition to amino groups might be modified or that cross-linking had occurred. We therefore undertook a more general study of the reaction of formaldehyde with proteins in the presence and absence of $NaBH_4$.

## MATERIALS AND METHODS

Grade I hen lysozyme, type III-A bovine pancreatic ribonuclease A, bovine serum albumin, BPN′ subtilisin, and *Streptomyces griseus* protease were from Sigma Chemical Co. Bovine trypsin (salt free, crystallized) and $\alpha$-chymotrypsin (twice crystallized) were obtained from Worthington Biochemical Co. The trypsin was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone according to Carpenter (1967) to inactivate any chymotrypsin present. Hammarstein quality casein was from Nutritional Biochemicals.

Trinitrobenzenesulfonic acid, recrystallized according to Fields (1972), and $NaBH_4$ were obtained from Sigma. Acetaldehyde and analyzed reagent grade 37% formaldehyde solution were from J. T. Baker Chemical Co. [$^{14}$C]Formaldehyde was from Schwarz/Mann and was diluted to a specific activity of 0.086 $\mu$Ci/$\mu$mol. All other chemicals were reagent grade.

Spectra were measured in a Cary Model 118-C spectrophotometer, using a thermostated sample cell holder at 25 °C. Optical densities were determined in a Beckman Model 25 spectrophotometer.

Protein concentrations were determined by weight of lyophilized samples and by optical density at 280 nm, using a value for $E_{1cm}^{1\%}$ of 26.3 for lysozyme and 7.3 for ribonuclease A. Counting of radioactivity was done in Aquasol (New England Nuclear Co.) using a Beckman LS-250 and Unilux II scintillation spectrometer. Counting efficiency was determined by an external standard, double-channel, ratio method and was always greater than 90%. Determinations of irreversibly bound (covalently linked) HCHO after reaction were made by direct calculation from the amount of radioactivity introduced after the treated samples had been separated from unreacted HCHO by dialysis against 1 mM HCl for at least 24 h with stirring. Concentrations of bound HCHO were also determined indirectly by a colorimetric method using chromotropic acid (Mitchell, 1953). In these determinations, the amounts of free (nonirreversibly bound) HCHO were measured by the chromotropic acid method and the bound HCHO calculated by difference.

Separations and estimations of polymeric forms of HCHO-treated lysozyme were done by molecular exclusion on Bio-Gel P-30 (Bio-Rad). Molecular sizes were estimated by comparisons with elution volumes for proteins of known molecular weights.

Reductive methylation of casein to achieve extensive modification was done according to Lin et al. (1969a). For less extensive methylation and ethylation, the milder procedures given by Means and Feeney (1968) and Rice and Means (1971) using less aldehyde were followed. In these procedures, the carbonyl compounds are always added after the reducing agent, $NaBH_4$, unless otherwise specified. Carbamoylation was done with KCNO (Chen et al., 1962; Means and Feeney, 1971) and acetylation with acetic anhydride (Means and Feeney, 1971).

The enzymatic activity of lysozyme was assayed turbidimetrically according to the method of Gorin et al. (1971), using dried cells of *Micrococcus lysodeikticus* (Worthington). The procedures of Canfield (1963) were followed for the hydrolysis of lysozyme with trypsin and fractionation of the resulting peptides on Dowex 50-X4 (J. T. Baker Co.).

The progress and extent of proteolytic activity were determined by measuring the number of amino groups liberated using trinitrobenzenesulfonic acid (Lin et al., 1969a) as modified by Fields (1972). This method was also used to measure the numbers of amino groups modified by alkylation, carbamoylation, and acetylation.

## RESULTS

**Extent of Formaldehyde Interaction with Ribonuclease and Lysozyme.** The amounts of HCHO irreversibly bound to lysozyme and ribonuclease were determined by counting the radioactively labeled HCHO bound after dialysis for at least 24 h against 1 mM HCl. The effects of varying the pH, temperature, time of in-

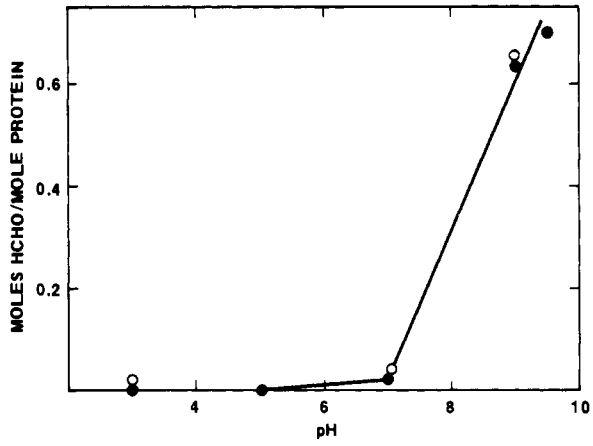


**Figure 1.** Effect of pH on the irreversible reaction of formaldehyde with lysozyme (●) and ribonuclease (○). $H^{14}CHO$ (0.17 mg) was added to 0.5 ml of a solution of the protein (10 mg/ml) in the appropriate 0.1 M buffer: sodium formate (pH 3), sodium acetate (pH 5), sodium phosphate (pH 7), or sodium carbonate (pH 9 and 9.5). The final calculated concentrations were 11.4 mM for HCHO, 0.68 mM for lysozyme, and 0.73 mM for ribonuclease. This gave a calculated value of approximately 16–17 for the HCHO:protein ratio. Incubation was at 21 °C for 30 min.

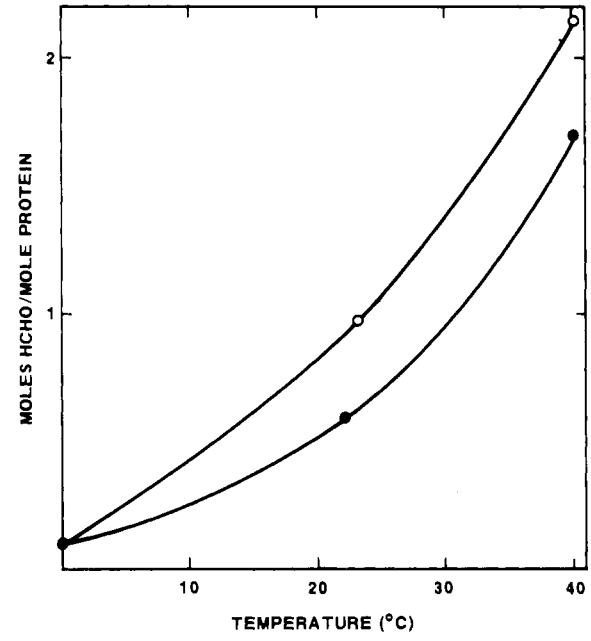


**Figure 2.** Effect of temperature on the irreversible reaction of formaldehyde with lysozyme (●) and ribonuclease (○). $H^{14}CHO$ (0.17 mg) was added to 0.5 ml of a solution of lysozyme (10 mg/ml) in 0.2 M borate buffer at pH 9.0, giving a calculated value of approximately 16 for the HCHO:lysozyme molar ratio. Incubation was for 30 min. The same buffer and incubation time were used for ribonuclease, but the $H^{14}CHO$ and protein concentrations were 0.66 and 2.5 mg/ml, respectively, giving a calculated value of approximately 120 for the HCHO:ribonuclease molar ratio.

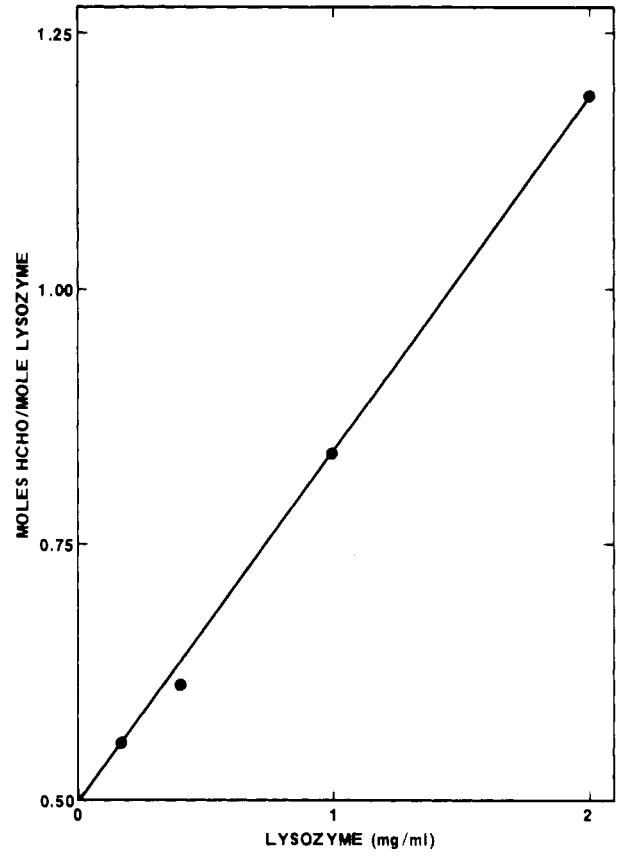


**Figure 3.** Effect of lysozyme concentration on its irreversible reaction with formaldehyde. $H^{14}CHO$ (0.17 mg) was added to 0.5-ml solutions of lysozyme at the indicated concentrations in 0.1 M, pH 9.0, borate buffer, at 21 °C for 6 h.

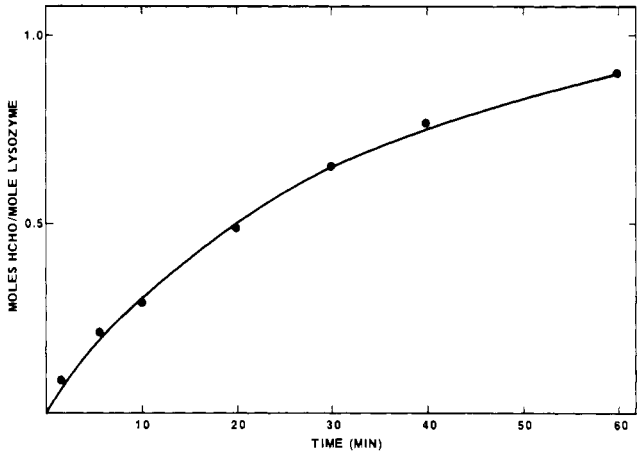


**Figure 4.** Effect of incubation time on the irreversible reaction of formaldehyde with lysozyme. $H^{14}CHO$ (0.17 mg) was added to 0.5 ml of solutions of lysozyme (10 mg/ml) in 0.1 M borate buffer at pH 9.0 and 21 °C.

cubation, concentration of protein, and concentration of formaldehyde are indicated in Figures 1–5.

At low concentrations of HCHO and HCHO to protein ratios of 16 to 17, lysozyme and ribonuclease reacted irreversibly at similar rates (Figure 1). The results of the effect of pH on the irreversible reaction with HCHO (Figure 1) agree with the known relative reactivity of HCHO for amino and other groups (Means and Feeney, 1971; Feeney et al., 1975). Although lysozyme undergoes a change in shape as the pH is lowered below 4 (Mohammadzadeh-K. et al., 1969), and an increase in reactivity might be expected on the basis of the concomitant exposures of groups in the protein, a much lower reactivity of amino groups, and possibly of other groups, would be expected at low pH.

Under the same conditions used for reductive alkylation (pH 9.0, 2 °C, for 30 min but without the addition of $NaBH_4$) less than 0.2 mol of HCHO per mol of protein was irreversibly bound (Figure 2). At temperatures as high as 40 °C (Figure 2) or incubation for as long as 6 h (Figures 3–5), less than 2 mol of HCHO per mol of protein was

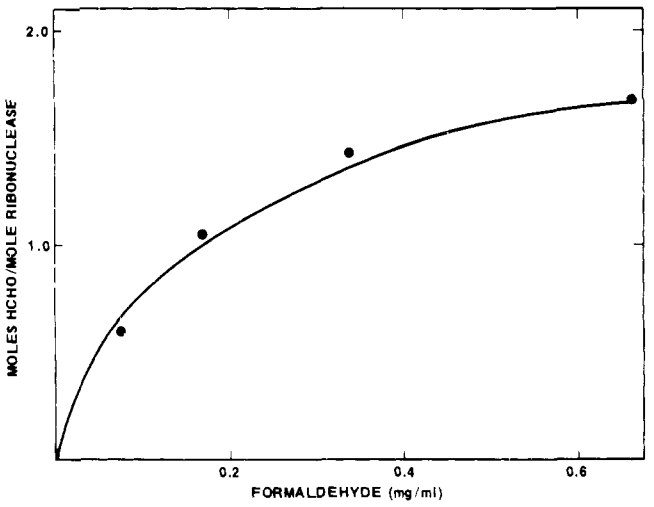


**Figure 5.** Effect of formaldehyde concentration on irreversible reaction with ribonuclease. The appropriate amounts of $H^{14}CHO$ were added to 0.5-ml solutions of ribonuclease (2.5 mg/ml) in 0.1 M borate buffer at pH 9.0 and 23 °C for 6 h.

bound. As shown by the data of Figure 4, the rate of the reaction is quite slow. Under the experimental conditions used, the extent of modification of lysozyme was directly proportional to protein concentration (0.2 to 2 mg/ml of lysozyme at 800- to 80-fold molar ratios of HCHO to protein) (Figure 3). Molar ratios of HCHO to protein greater than 100 caused little additional irreversible binding of HCHO to ribonuclease (Figure 5).

These results indicated that under the mild conditions used for reductive alkylation, much longer times would have been necessary for extensive (greater than 1 mol of HCHO/mol of protein) irreversible bindings to occur.

Comparisons were made of the reaction of formaldehyde with casein by determining the amount of radioactive formaldehyde bound and by determining the unreacted formaldehyde with chromotropic acid. Serial determinations were made in a rate assay over a period of 20 min. Within the limits of experimental error, similar amounts of formaldehyde were determined as being bound by both procedures (approximate accumulated error ±10%).

**Estimation of Formaldehyde Available to Nonspecifically Interact during Reductive Alkylation.** The introduction of greater than 1 mol of HCHO per mol of lysozyme within a few hours in the absence of $NaBH_4$ indicated that this reaction might compete to a greater extent with reductive alkylation than had previously been supposed (Means and Feeney, 1968). Since the reductive alkylation procedure is done over a 30-min period with concentrations of protein similar to those in Figures 1–5, it might be possible to introduce as much as 1 mol of HCHO by nonreductive pathways, providing that the formaldehyde concentration would be sufficiently high for a long enough time to interact nonspecifically. The possible presence of residual HCHO was determined by the chromotropic acid procedure after the addition of each increment of HCHO during reductive alkylation. Within the limits of time that reagents could be conveniently added for analysis, less than 5% of the HCHO was present in less than 4 s after its addition.

**Effects of Formaldehyde Binding on Properties of Lysozyme.** The reaction of formaldehyde (without $NaBH_4$) with lysozyme at pH 9 and room temperature for 6 h resulted in the formation of aggregates. This was observed visually and turbidimetrically, as well as by fractionation of the products and estimation of the physical

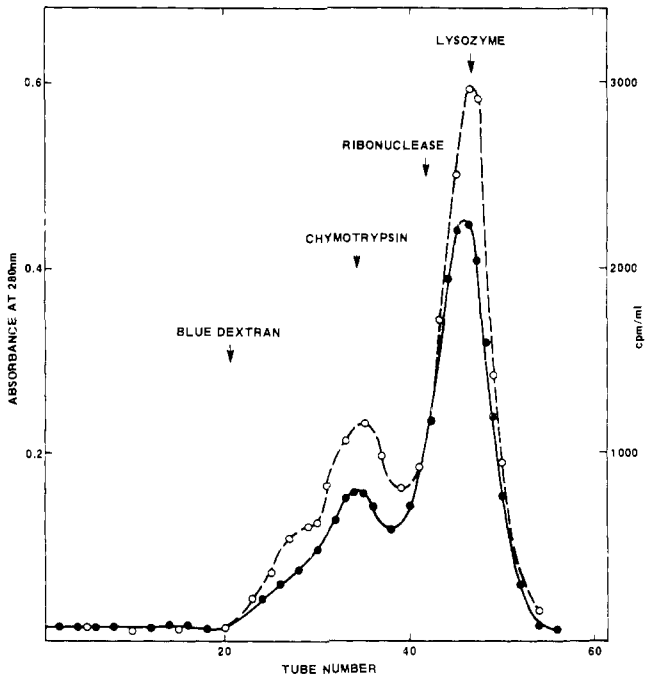


**Figure 6.** Fractionation of formaldehyde-treated lysozyme on Bio-Gel P-30. Three and one-half milliliters of a solution of lysozyme (2.5 mg/ml) and $H^{14}CHO$ (2.6 mg/ml) in 0.2 M borate buffer at pH 9.1 was incubated at 21 °C for 6 h. The calculated molar ratio of HCHO to lysozyme was approximately 500. An aliquot of this sample was dialyzed against 0.001 M HCl for 24 h and against $H_2O$ for 12 h. It was then put through a column of Bio-Gel P-30. The elution profiles are of counts per min (○) and absorbance (●). The elution volumes of standard proteins determined in separate runs are shown on the figure.

sizes. In control solutions of lysozyme (0.7 mg/ml) in formic acid (0.1 M) or in borate buffer (0.2 M, pH 9.0), no changes in enzymatic activity or in turbidity were detected on incubation over a 6-h period at room temperature. When the lysozyme solution in borate buffer was made 5 mM in HCHO, however, there was a progressive development of turbidity during this 6-h period. More concentrated solutions of lysozyme and HCHO developed extensive precipitates under the same conditions.

This polymerization was studied on a quantitative basis by fractionation of formaldehyde-treated samples on Bio-Gel P-30 (Figure 6). The higher molecular weight fractions were more highly labeled; the HCHO:lysozyme ratios were 1.5 (±0.2):1 and 1.3 (±0.1):1 for the polymeric (mainly dimers) and monomeric fractions, respectively. In a second experiment designed for more extensive modification, incubation was for 8 h rather than 6 h. Higher molecular weight species were more predominant, and components appearing to be dimers and trimers, as well as larger polymers, were easily seen. In all experiments the monomeric form was nearly 100% enzymatically active, whereas the higher molecular weight species were less active. Protein eluted first from the column had no detectable activity, but the dimers and trimers had approximately 30–40% activity of the control.

**Peptide Separation of Formaldehyde-Treated Lysozyme.** Lysozyme was treated with radioactively labeled HCHO and fractionated on Bio-Gel P-30, as described in Figure 6. The monomeric fraction was dialyzed and lyophilized. It was then reduced with 2-mercaptoethanol, alkylated with iodoacetic acid, and hydrolyzed with trypsin for 5 h at 37 °C and pH 7.5. The hydrolysate was chromatographed on a 0.7 × 120 cm column of Dowex 50-X4.

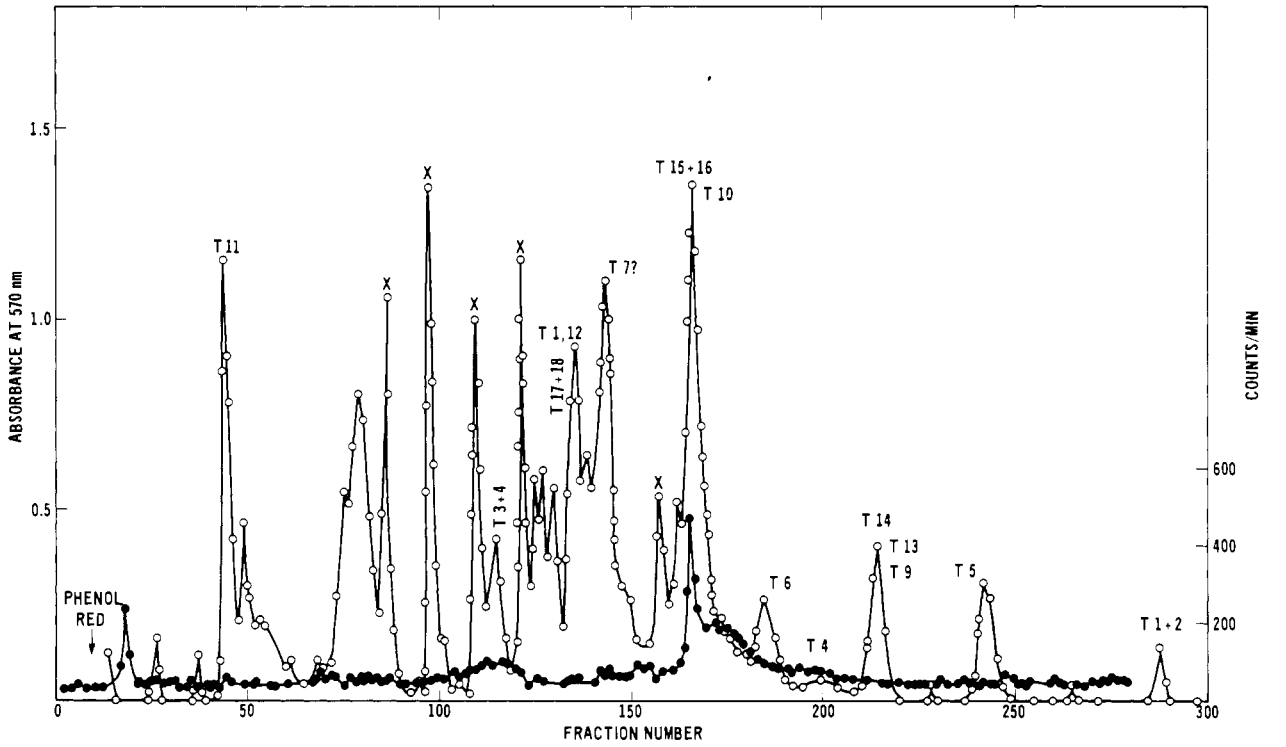


**Figure 7.** Fractionation of peptides from trypsin treatment of formaldehyde-treated lysozyme. A solution of lysozyme (2.5 mg/ml) and $H^{14}CHO$ (2.6 mg/ml) in 0.2 M borate buffer at pH 9.1 was incubated at 21 °C for 6 h (same sample as in Figure 6). The sample was then dialyzed against 0.001 M HCl for 24 h and $H_2O$ for 12 h. It was then put through a column of Bio-Gel P-30 and the monomer fraction (tubes 42–55) (see Figure 6) was lyophilized. This product was reduced, alkylated with iodoacetic acid, digested with trypsin, and chromatographed through a 0.7 × 120 cm column of Dowex 50-X4. Elution was according to the method of Canfield (1963). The $^{14}C$ content (●) of aliquots of each eluted sample was counted and aliquots were reacted with ninhydrin, and the absorbancies at 570 nm were determined (○). T 1, T 2, ... T*n*, peptides identified from Canfield (1963); X, peptides not identified and absent from control.

The entire process, including the elution scheme, was done according to the method of Canfield (1963). The eluates were analyzed and collected by splitting the stream, one part through the ninhydrin detection system of a Technicon amino acid analyzer, and the other collected in 10-ml volumes on a fraction collector. Samples from the individual tubes were counted for radioactivity. Controls of lysozyme without added HCHO were treated identically except for the counting of radioactivity.

Ninhydrin analysis of the elution profiles revealed that many of the peaks from the HCHO-treated sample could be assigned without ambiguity on comparison with patterns from the control sample and from the previously published patterns of Canfield (1963) (Figure 7). There were, however, several peaks that were absent from the control and previously published patterns. Small amounts of radioactivity were scattered among several fractions, but the major amount was spread through fractions 130 to 180.

**Proteolysis of Reductively Methylated Proteins.** When extensively reductively methylated (greater than 90% of the amino groups) casein was hydrolyzed with α-chymotrypsin, both the rate and extent of hydrolysis were less than half those obtained with the original casein. When only 40–45% of the amino groups were reductively methylated, the decrease in extent of hydrolysis was not as large. Still lower extents of modifications caused smaller but significant decreases (Figure 8). Since α-chymotrypsin has very little activity at lysine residues, our initial conclusion was that the lowered susceptibility was caused by nonspecific modification, i.e., derivatization of residues normally susceptible to α-chymotrypsin or the formation of cross-links. But that such was not the cause of the smaller hydrolysis was indicated by two additional types of experimental data. (a) When the reductive methylation procedure was done without addition of $NaBH_4$, there was

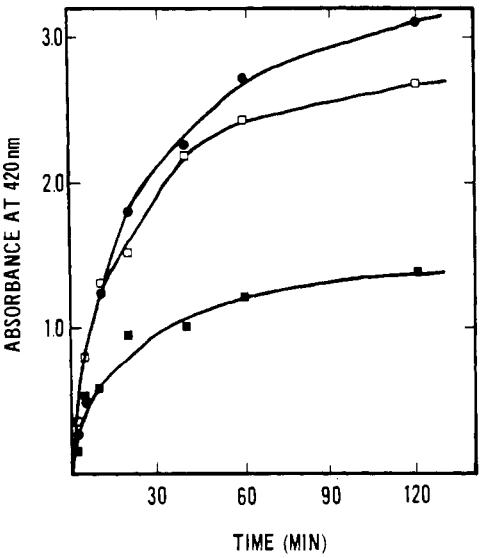


**Figure 8.** Digestion of casein and chemically modified caseins by bovine α-chymotrypsin. Amino groups of casein were reductively methylated and the hydrolysis by α-chymotrypsin followed with time. Unmodified casein (●), reductively methylated casein–33% modified (□), reductively methylated casein–52% modified (■). The casein concentrations were 0.2 mg/ml in 0.02 M borate buffer at pH 8.2 and the α-chymotrypsin was 3.2 μg/ml. The hydrolysis was followed by the procedure of Lin et al. (1969a). Δ absorbance was based on changes in absorbance per milligram of substrate.

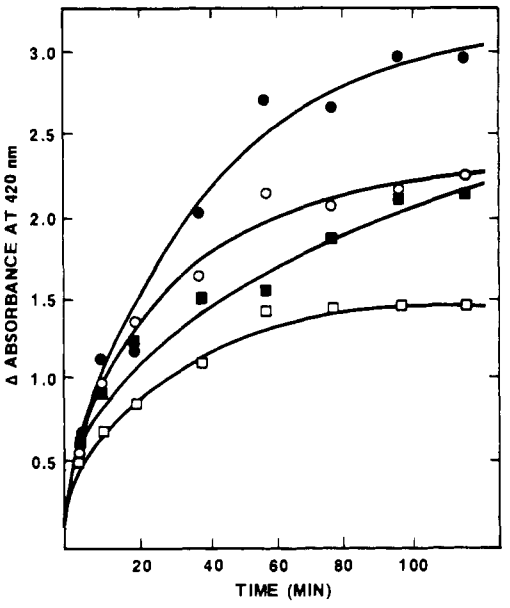


**Figure 9.** Chymotryptic hydrolysis of bovine serum albumin (BSA) modified by N-dimethylation. The BSA was reductively methylated to various extents with HCHO and $NaBH_4$ according to Means and Feeney (1968): (●) unmodified, (○) 37% modified, (■) 69.5% modified, and (□) 86% modified. The products (0.2 mg/ml in 0.02 M borate buffer, pH 8.2) were incubated with 3.2 μg/ml of α-chymotrypsin for the times indicated. Proteolysis was followed according to Lin et al. (1969a) using Field's (1972) modification of the trinitrobenzenesulfonic acid assay. Δ absorbance was based on changes in absorbance per milligram of substrate.

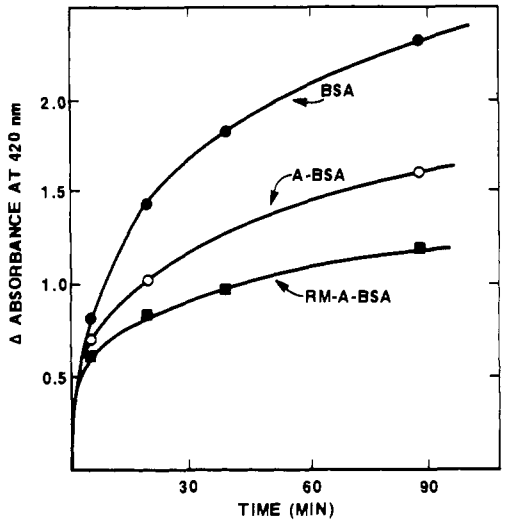


**Figure 10.** Chymotryptic hydrolysis of denatured bovine serum albumin (BSA) modified by partial N-acetylation and partial N-acetylation plus N-dimethylation. BSA (●) was denatured by heating. Aliquots were then acetylated [A-BSA (○)] and doubly modified by partial acetylation followed by reductive methylation [RM-A-BSA (■)]. The products (0.2 mg/ml in 0.02 M borate buffer, pH 8.2) were incubated with 3.2 μg/ml of α-chymotrypsin for the times indicated. Hydrolysis was followed according to Lin et al. (1969a) using Field's (1972) modification of the trinitrobenzenesulfonic acid assay. Δ absorbance was based on changes in absorbance per milligram of substrate.

no detectable decrease in hydrolysis of casein. This eliminated nonreductive modification by the formaldehyde as the cause. (b) When acetaldehyde was substituted for the formaldehyde, the reductively ethylated casein underwent a decrease in hydrolysis by α-chymotrypsin similar to that obtained with reductively methylated casein. This eliminated the unusual reactivity of formaldehyde in side reactions (Feeney et al., 1975) as the cause.

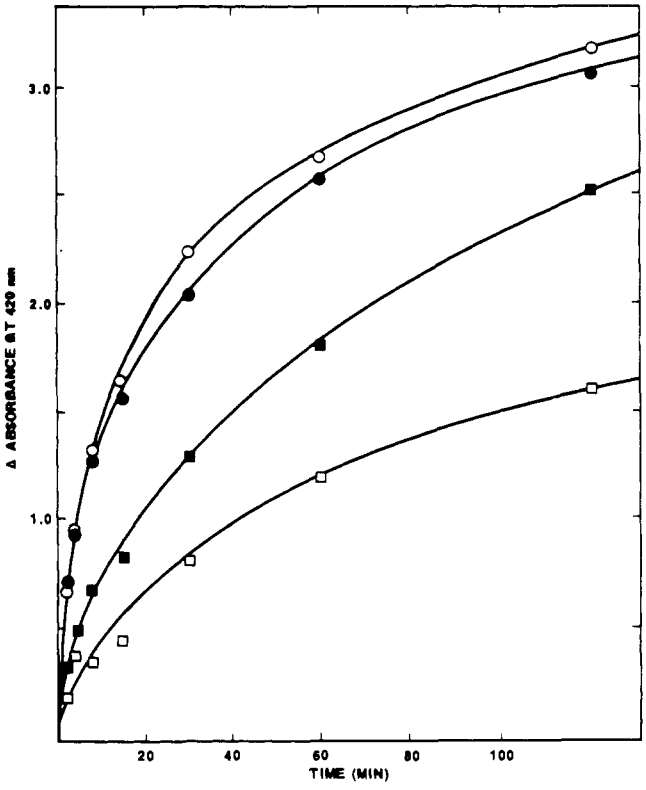


**Figure 11.** Inhibitory effects of peptides from chymotryptic hydrolysates of modified bovine serum albumin (BSA) on hydrolysis of BSA by α-chymotrypsin. BSA was acetylated and then reductively methylated as described in the text. Eight milliliters (0.87 mg/ml) of this product in 0.012 M Tris buffer and 0.012 M $CaCl_2$ (pH 7.7) was incubated at 37 °C for 24 h with 200 μg of α-chymotrypsin. The solution was then boiled for 10 min. Aliquots of this mixture of peptides were then added in the indicated proportions of BSA to the peptides. The various ratios of BSA to peptides in assay were: 17:1 (●); 3.5:1 (■); 1.7:1 (□); BSA alone (○). The BSA concentration was 0.2 mg/ml in 0.02 M borate buffer at pH 8.2 and the α-chymotrypsin was 3.2 μg/ml. The hydrolysis was followed according to Lin et al. (1969a) using Field's (1972) modification of the trinitrobenzenesulfonic acid assay. Δ absorbance was based on changes in absorbance per milligram of substrate.

Further studies were done with bovine serum albumin because of its relatively greater number of amino groups. The decreased rates and extents of hydrolyses by α-chymotrypsin of bovine serum albumin reductively methylated to different extents (Figure 9) were approximately the same as those found with reductively methylated casein. Partial acetylation, partial acetylation followed by reductive methylation, partial carbamoylation, and partial carbamoylation followed by reductive methylation resulted in decreases in the extent of hydrolysis proportional to the extent of modification (Figure 10). The effect was also not related to the degree of nativity of the protein, as reductively methylated, denatured bovine serum albumin also evidenced decreases in hydrolysis. The effect was thus not unique to casein nor to reductive methylation.

Previous observations from this laboratory had indicated that substrate inhibition was not a major factor in hydrolysis of reductively methylated proteins by α-chymotrypsin (Lin et al., 1969a). In seeking other causes of the

lowered susceptibility, product inhibition was found to be a major contributing factor (Figure 11). When peptides prepared by chymotryptic hydrolysis of extensively methylated bovine serum albumin were added together with unmodified bovine serum albumin, the rate of hydrolysis was decreased about two-thirds. In control experiments in which chymotryptic hydrolysates of nonmethylated bovine serum albumin were added, the decreases in rates of hydrolysis were less than 20% of those noted with the peptides from the methylated bovine serum albumin.

## DISCUSSION

The principal initial reactions of HCHO with proteins under mild conditions and without reduction are reversible (Means and Feeney, 1968; Feeney et al., 1975). *N*-Hydroxymethyl derivatives are formed with the ring nitrogens of histidine (Dunlop et al., 1973; Martin et al., 1975), the $\epsilon$-amino groups of lysine, and the $\alpha$-amino groups of $NH_2$-terminal amino acids (Means and Feeney, 1968; Feeney et al., 1975). Sulfhydryls, when present, also may react. With amino groups, both a monohydroxymethyl and a dihydroxymethyl adduct may be formed (Means and Feeney, 1968). These reactions are very rapid, and the adducts are quickly dissociated by the removal of the reagent. However, small amounts of imines (Schiff bases) are also present in the equilibrium mixture with amino groups, and these would be rapidly reduced to the methyl derivatives by $NaBH_4$, thereby shifting the equilibrium and rapidly driving the reaction to completion.

Little is apparently known about the mechanisms of the much slower irreversible reactions of HCHO with proteins. Many of these probably go through imine intermediates. Cross-links between tyrosines and lysines (Bizzini and Raynaud, 1974; Warren et al., 1974) or between amides and other groups (Myers and Hardman, 1971) could form polymers. Extensive treatments of casein with HCHO produced such drastic changes that the product was indigestible when fed to sheep (Reis and Tunks, 1973).

The results of our study confirm that the irreversible reaction of HCHO with proteins at room temperature and neutrality is comparatively slow. Several hours, for example, were required for the introduction of as little as 2 mol of HCHO/mol of protein into lysozyme, which eventually resulted in the formation of higher polymers. These observed effects on lysozyme suggest that the losses of activity reported by Alderton et al. (1946) might have been accompanied by, or caused by, polymerization. The inactivation of ribonuclease A by HCHO reported by Zittle (1948) was previously confirmed in our laboratory (Means and Feeney, 1968). The initial rate of inactivation was very rapid (approximately 40% in less than 1 min) under conditions similar to those used in the present study, and was followed by a slower rate of activity loss (Means and Feeney, 1968). The initial rate of inactivation was proportional to the amount of HCHO added, and was reversed upon dilution and short incubation. The half-life of this reversible inactivation was reported to be similar to that for the formation of the corresponding hydroxymethyl derivatives from HCHO and either butylamine or bovine serum albumin (Means and Feeney, 1968). The slower rate of irreversible inactivation and covalent binding found in this study (Figure 4) appeared to be the same as the slow rate of irreversible inactivation previously reported (Means and Feeney, 1968). In contrast to lysozyme, ribonuclease has an amino group essential for activity (Means and Feeney, 1968). The fast reversible reaction of ribonuclease may involve this amino group, which is probably the one that is modified by reductive alkylation, causing loss of activity. Further characterization of the slow, irreversible reactions, which can involve the formation of polymers and changes in patterns of peptides after proteolysis (Figure 7), is obviously indicated (Means and Feeney, 1971; Myers and Hardman, 1971; Bizzini and Raynaud, 1974; Warren et al., 1974; Feeney et al., 1975).

The procedure of reductive alkylation was designed and developed to eliminate the possibility of the slow nonspecific side reactions (Means and Feeney, 1968; Feeney et al., 1975). In the reductive alkylation procedure, the reducing agent and the protein are first mixed before addition of the carbonyl compound expressly for this purpose. The relative concentrations used are such that the initial carbonylamine interaction products are very rapidly reduced to the alkyl compounds by $NaBH_4$. The technique was designed not only to give a rapid specific reaction, but also to remove HCHO in equilibrium before it has the opportunity to engage in less rapid side reactions. For this reason, no polymers of ribonuclease or lysozyme are observed even with extensive alkylation. Also, no changes in absorbance or in amino acid composition after acid hydrolysis (other than the formation of alkylated amino groups) were observed. This was in part confirmed by the results of the chromotropic acid analyses in the present study which showed that addition of the reducing agent before the carbonyl compound not only rapidly produces the methylamine derivatives, but also quickly removes excess HCHO by reducing it to methanol. The nonspecificity after extensive reductive alkylation reported by Ottesen and Svensson (1971) might have been due to exposure of the protein to HCHO before addition of $NaBH_4$. Our results confirm our previous recommendations for first adding the reducing agent (Means and Feeney, 1968).

Our observation that the decreased chymotryptic hydrolysis of reductively alkylated proteins was due to product inhibition rather than nonspecific modification suggests that inhibition by chemically modified proteins may be more common than heretofore suspected. Further studies of the effects of modified proteins on enzyme inhibition are underway in our laboratory.

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## LITERATURE CITED

Alderton, G., Fevold, H. L., Lightbody, H. D., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **5**, 119 (1946).
Bizzini, B., Raynaud, M., *Biochimie* **56**, 297 (1974).
Canfield, R. E., *J. Biol. Chem.* **238**, 2691 (1963).
Carpenter, F. H., *Methods Enzymol.* 11, 237 (1967).
Chen, C. C., Grossberg, A. L., Pressman, D., *Biochemistry* 1, 1025 (1962).
Dunlop, P., Marini, M. A., Fales, H. M., Sokoloski, E., Martin, C. J., *Bioorg. Chem.* **2**, 235 (1973).
Feeney, R. E., Blankenhorn, G., Dixon, H. B. F., *Adv. Protein Chem.* **29**, 135 (1975).
Fields, R., *Methods Enzymol.* **25B**, 464 (1972).
Gorin, G., Wang, J. F., Papavlou, L., *Anal. Biochem.* **39**, 113 (1971).
Grisolia, S., Guerri, C., Godfrey, W., *Biochem. Biophys. Res. Commun.* **66**, 1112 (1975).
Hemsley, J. A., Reis, P. J., Downes, A. M., *Aust. J. Biol. Sci.* **26**, 961 (1973).
Lin, Y., Means, G. E., Feeney, R. E., *J. Biol. Chem.* **244**, 789 (1969a).
Lin, Y., Means, G. E., Feeney, R. E., *Anal. Biochem.* **32**, 436 (1969b).
Martin, C. J., Lam, D. P., Marini, M. A., *Bioorg. Chem.* **4**, 22 (1975).

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).

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# Effects of Alkali on Proteins. Disulfides and Their Products

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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 $\alpha$-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 $\beta$ elimination of the disulfides resulting in the intermediate, dehydroalanine. Michael-type nucleophilic additions of the $\epsilon$-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 $\beta$-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, $\Delta F^*$, 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) $\alpha$ elimination which involves the initial abstraction of proton on a carbon $\alpha$ 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) $\beta$ elimination which involves abstraction of the acidic proton from a carbon $\beta$ 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 $\alpha$-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-

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