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Degradation of protein disulphide bonds in dilute alkali

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The degradation of S–S bonds in 0.2 M-NaOH at 25°C was studied for a series of proteins and simple aliphatic disulphide compounds, by using cathodic stripping voltammetry, ion-selective-electrode potentiometry, spectrophotometry and ultrafiltration. The disulphide bonds that dissociated in 0.2 M-NaOH were usually those that are solvent accessible and that can be reduced by mild chemical reductants. Some unexpected differences were found between similar proteins, both in the number of S–S bonds dissociated and in their rates of decomposition. Chymotrypsin has one S–S bond attacked, whereas chymotrypsinogen and trypsinogen have two. Ribonuclease A has two S–S bonds dissociated, but ribonuclease S and S-protein have three. Denaturation in 6 M-guanidine hydrochloride before alkaline digestion caused the loss of an additional S–S bond in ribonuclease A and insulin, and increased the rate of dissociation of the S–S bonds of some other proteins. The initial product of S–S bond dissociation in dilute alkali is believed to be a persulphide intermediate formed by a β-elimination reaction. This intermediate is in mobile equilibrium with bisulphide ion, HS^-, and decomposes at a mercury electrode or in acid solution to yield a stoichiometric amount of sulphide. Rate constants and equilibrium constants were measured for the equilibria between HS^- and the intermediates involved in the alkaline dissociation of several proteins. Elemental sulphur was not detected in any of the protein digests. It is suggested that formation of HS^- from a persulphide intermediate involves a hydrolysis reaction to yield a sulphenic acid derivative. The small polypeptides glutathione and oxytocin gave only a low yield of persulphide, and their alkaline decomposition must proceed by a mechanism different from that of the proteins.

Protein S–S bonds serve as structural elements and stabilize the folded structure of the molecule. An S–S bond can bridge distant regions of a polypeptide chain to form a unit of structure known as a 'disulphide loop' (Tamburro *et al.*, 1970). Although an S–S group may not be a component of the active centre of an enzyme, S–S bonds are often essential for maintaining the conformation and rigidity of enzyme active centres, and of critical regions of other proteins (Knights & Light, 1976). Destruction of these essential S–S bonds invariably leads to loss of enzymic activity.

Whereas some enzymes, e.g. insulin and chymotrypsin, are completely inactivated by the reduction of a single S–S bond (Wold, 1971; Martin & Viswanatha, 1975), others can retain full or partial activity with some of these bonds reduced. These 'non-essential' S–S bonds are not randomly selected,

Abbreviations used: c.s.v., cathodic stripping voltammetry; GSSG, oxidized glutathione.

but are particular bonds in the peptide sequence, and are usually near the surface of the molecule or are readily accessible to chemical reductants like borohydride or dithiothreitol. Free radicals produced in solution by ionizing radiation rapidly attack S–S bonds, which are by far the most radiolabile groups in a protein (Risi *et al.*, 1967; Adams, 1972). The S–S bonds first destroyed are those that are also most susceptible to chemical reduction.

It is well established that sulphur-rich proteins are degraded in even mildly alkaline solution to form a variety of products, losing about half of their sulphur as H_2S (Danehy, 1966). Amongst the products are lanthionine, lysinoalanine and ornithinoalanine (Ziegler *et al.*, 1967). Lysinoalanine, formed from lysine and dehydroalanine residues produced by alkaline dissociation of protein cystine or glycolically-bound seryl groups (Bohak, 1964), has recently been shown to be carcinogenic in rats (Sternberg *et al.*, 1975). Lysinoalanine also de-

creases protein utilization and digestibility, and was measured at relatively high concentrations in a wide range of commercial food products, including protein concentrate and dried egg powder (Sternberg *et al.*, 1975).

The mechanism of alkaline degradation of proteins has been investigated by several workers (Danehy, 1966; Nashef *et al.*, 1977a). There is still considerable dispute, however, about the reaction paths and why free cystine and cystine in a peptide linkage behave completely differently towards alkali. In an earlier publication (Florence, 1979b) it was shown that, for a variety of proteins, the number ($\bar{n}$) of S–S bonds dissociated in 0.2 M-NaOH at 25°C was similar to the number attacked by mild chemical reductants. The reaction in 0.2 M-NaOH was followed by measuring sulphide ion by using cathodic stripping voltammetry (c.s.v.), and by isothermal distillation to determine total H_2S liberated when the solution was acidified. The present paper deals with the mechanism of alkaline degradation, and shows that different $\bar{n}$ values are obtained for such closely related proteins as ribonuclease A and S, and chymotrypsin and chymotrypsinogen. Ion-selective-electrode potentiometry was used to demonstrate that the equilibrium concentration of free sulphide ion in an alkaline protein digest is less than that measured by c.s.v. or acid distillation, and that HS^- is in mobile equilibrium with another sulphur compound, probably a labile persulphide intermediate.

Experimental

Materials

Data for the proteins studied are listed in the order: protein and source, supplier, $A_{1cm}^{1\%}$, and analytical wavelength (nm) used to determine the purity. Albumin, bovine serum, Sigma, 6.6 (278); α-chymotrypsin A, bovine, Calbiochem A grade, 20.4 (280); α-chymotrypsinogen A, bovine, Sigma type II, 20.4 (280); deoxyribonuclease, bovine, Sigma, 11.4 (280); insulin, bovine, Sigma, 11.0 (277); lipoamide dehydrogenase, pig, Sigma type III, 11.8 (273); lysozyme, egg white, Sigma grade I, 26.5 (282); ovomucoid, egg white, Sigma type II, 4.10 (278); papain, papaya, Sigma, 24.9 (280); pepsin, pig, Sigma, 14.5 (280); rhodanese, bovine liver, Sigma type I, 17.5 (280); ribonuclease A, bovine, Sigma type III-A, 7.3 (280); ribonuclease S, bovine, Sigma type XII-S, 7.3 (280); ribonuclease S-protein, bovine, Sigma grade XII-PR, 7.8 (280); superoxide dismutase, bovine, Sigma, 3.16 (258); trypsinogen, bovine, Worthington, 14.0 (280); urease, jackbean, Sigma type IX, 5.57 (278). The purity of oxytocin, Sigma grade V, 10% (w/w) in mannitol, was determined by the biuret method. Superoxide dismutase, apoprotein, was prepared

from the native enzyme by dialysis against acetate/EDTA, pH 3.8 (Abernethy *et al.*, 1974).

The 0.2 M-NaOH supporting electrolyte was prepared from Merck 'Suprapur' NaOH solution, and was standardized against acid. All other reagents were A.R.-grade quality. Standard sulphide solutions were prepared daily from $Na_2S,9H_2O$ that had been analysed iodimetrically. Mercury was purified as described previously (Florence, 1979a). High purity N_2 was passed through an Alltech O_2 trap, then through an in-line membrane filter before entering the electrochemical cell.

Cathodic stripping voltammetry

C.s.v. was used to follow the production of sulphide and sulphide-liberating compounds during the digestion of proteins (5–40 $\mu g \cdot ml^{-1}$) in N_2-deaerated 0.2 M-NaOH at 25.0 ± 0.5°C. The technique involved (Florence, 1979a,b) electrochemical deposition of sulphide ion from a stirred solution onto a mercury electrode held at −0.35 V versus saturated calomel electrode. After a suitable deposition period (0.5–2 min) the HgS film was stripped from the electrode by applying a −0.35 V to −1.0 V d.c. ramp at 83 $mV \cdot s^{-1}$. The current peak at −0.79 V versus saturated calomel electrode due to stripping of the HgS was measured after various times until the height remained constant. Sulphide was then determined by several additions of standard sulphide solution to the c.s.v. cell and re-measurement of the sulphide peak.

Determination of sulphide with the sulphide-ion-selective electrode

Proteins affect the response of the sulphide-ion-selective electrode, but this problem was overcome by the use of standard additions of relatively high concentrations of sulphide, or by using a Gran plot to determine the initial concentration of sulphide (Bailey, 1976). Thiols such as mercaptoacetic acid, cysteine and reduced glutathione did not affect the response of the electrode at molar concentrations 10-fold higher than that of the protein (Tseng & Gutknecht, 1975).

Ion-selective electrode measurements of the proteins (0.5×10^{-4} M–1.0×10^{-4} M) were made in deaerated 0.2 M-NaOH at 25.0 ± 0.5°C, and the electrode potential of the Orion sulphide-ion-selective electrode was recorded as a function of time while the solution was kept under a blanket of N_2. The electrode surface was cleaned and polished before each experiment.

Other procedures

Spectrophotometric measurements were made at 25.0 ± 0.2°C with a Hitachi u.v.-visible recording spectrophotometer and a Cary 16 manual high-precision spectrophotometer. All solutions were

deaerated with N_2 before use, and the cuvettes were tightly stoppered. For calculating first-order rate constants from spectrophotometric kinetic data when the final absorbance was unknown, the graphical procedure of Schwartz & Gelb (1978) was used.

Ultrafiltration of bovine serum albumin solutions in 0.2 M-NaOH was performed under N_2 pressure with an Amicon Diaflo PM 30 membrane (greater than 95% rejection for mol.wt. greater than 60000). A new membrane was used for each experiment.

Elemental sulphur in protein digests was measured by extraction into n-heptane. A 5 ml aliquot of the protein solution in 0.2 M-NaOH was mixed with 0.9 ml of 1.0 M-HCl and 1.0 ml of 0.2 M-phosphate buffer, pH 8.3. The solution was shaken for 10 min with 10.0 ml of n-heptane. The emulsified organic layer was filtered through a Whatman 1S phase separation paper. The clear n-heptane filtrate was washed by shaking with 5 ml of 0.01 M-NaOH, then filtered again through a Whatman 1S paper. The absorbance was measured at 280 nm versus n-heptane carried through a blank procedure. The molar absorption coefficient of sulphur in n-heptane at 280 nm is 787 litre·mol⁻¹·cm⁻¹. Aliquots of a suspension of hydrophilic colloidal sulphur (Roy & Trudinger, 1970), carried through the extraction procedure in the presence of bovine serum albumin, were recovered completely.

The spectrofluorimetric determination of sulphide and thiol by depression of the fluorescence of fluorescein mercuric acetate was carried out by a modification of the procedure of Karush et al. (1964). An aliquot of the test solution (1–7 μg of S) was added to a 25 ml volumetric flask, followed by 2.5 ml of 0.5 M-Tris/HCl buffer, pH 7.5, and 0.10 ml of fluorescein mercuric acetate solution [0.2% (w/v) in 1 M-NaOH]. The solution was diluted to volume with water and mixed. The fluorescence was measured with an excitation wavelength of 489 nm and emission wavelength of 520 nm, setting the blank at a reading of 100 on the Perkin–Elmer model 203 fluorimeter. The concentration of sulphur compound was read against a calibration curve. A reading of 70 was obtained for 3 μg of sulphur added as sulphide.

Results

Cathodic stripping voltammetry

In dilute NaOH supporting electrolyte, c.s.v. is highly selective for sulphide ion. Very few compounds produce a stripping peak in this medium (Florence, 1979a,b) and, for those that do, the peak potential is more positive than that for sulphide (−0.79 V versus saturated calomel electrode). The reactions are:

Deposit (i_d): $HS^- + OH^- + Hg \rightarrow HgS + H_2O + 2e^-$

$$(1)$$

Stripping (i_p): $HgS + 2e^- \rightarrow Hg + S^{2-}$ (2)
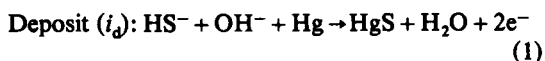
Cysteine and other thiols yielded very insensitive peaks, with potentials in the vicinity of −0.55 V versus saturated calomel electrode. All S–S-containing proteins gave c.s.v. peaks in 0.2 M-NaOH that had the peak potential, shape and other characteristics of the sulphide ion (Fig. 1). Table 1 shows results for the number $(\bar{n})$ of protein S–S bridges cleaved (on the assumption that one mole of sulphide or sulphide-liberating compound is produced per S–S bridge dissociated), as well as the first-order rate constants in 0.2 M-NaOH for the growth of the c.s.v. wave. For most of the proteins, first-order kinetics were obeyed to at least 85% completion of reaction. Included in Table 1 are new, more precise results for some of the proteins previously studied (Florence, 1979b). All of the proteins gave $\bar{n}$ values that were integral numbers within the precision of the method. This strongly suggests that particular S–S bonds are broken in 0.2 M-NaOH.

The effect of a preliminary denaturation of the proteins in 6 M-guanidine hydrochloride was also investigated. A stock solution of protein $(2 \times 10^{-4} M)$ was incubated in 6 M-guanidine hydrochloride for

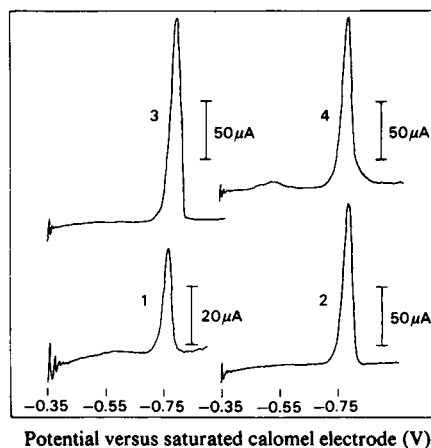Potential versus saturated calomel electrode (V)

Fig. 1. C.s.v. curves

Current–potential curves were obtained at 25°C by using a c.s.v. cell with a mercury-pool working electrode, a deposition potential of −0.35 V versus saturated calomel electrode and a scan rate of 83 mV·s⁻¹. Curve 1, 1.65×10^{-7} M-trypsinogen in deaerated 0.2 M-NaOH after 4.0 min digestion (1 min deposition); curve 2, as for curve 1, but after 40 min digestion (1 min deposition); curve 3, as for curve 1, but after 122 min digestion and $+3.05 \times 10^{-7}$ M-HS⁻ (0.5 min deposition); curve 4, 7.05×10^{-7} M-cysteine persulphide $(1.37 \times 10^{-6}$ M-cysteine $+ 7.05 \times 10^{-7}$ M-elemental sulphur) in deaerated 0.2 M-NaOH (1 min deposition).

Table 1. *Measurement of the cleavage of protein and peptide S–S bridges in 0.2 M-NaOH at 25°C by using c.s.v.*
The number of S–S bridges cleaved ($\bar{n}$) was calculated from mol of HS^- released/mol of protein, as measured by c.s.v. The $\bar{n}$ results show the means and relative S.D. and the number of separate determinations (in parentheses). The first-order rate plots sometimes showed two intersecting straight line portions; the $\bar{n}$ value at the point of intersection is shown. The molecular weights and total number of S–S bridges are values from the recent literature.

Protein or peptide used for c.s.v.	Concn. (μg/ml)	Mol.wt.	Total no. of S–S bridges	No. of S–S bridges cleaved ($\bar{n}$)	$10^2 \times$ First-order rate constant in 0.2 M-NaOH, k_1 (min^{-1})
Albumin, bovine	10	66 500	17	5.2 ± 0.3 (7)	1.12
α-Chymotrypsin A	10	24 500	5	1.10 ± 0.06 (5)	3.0
α-Chymotrypsinogen A	10	25 000	5	1.91 ± 0.08 (5)	0.44, 1.68 ($\bar{n} = 0.96$)
Deoxyribonuclease	20	31 000	2	0.95 ± 0.00 (2)	1.12
Glutathione, oxidized	5	612	1	0.076 ± 0.003 (3)	4.3
Insulin	5	5733	3	0.98 ± 0.03 (9)	1.22
Lipoamide dehydrogenase	20	100 000	4	1.8 ± 0.2 (2)	2.7, 0.4 ($\bar{n} = 0.98$)
Lysozyme	5	14 400	4	3.1 ± 0.2 (4)	0.21, 2.2 ($\bar{n} = 1.05$)
Ovomucoid	5	27 500	8	4.2 ± 0.2 (2)	3.0
Oxytocin	5	980	1	0.072 ± 0.005 (5)	0.83
Papain	15	20 900	3	0.96 ± 0.06 (4)	3.7
Pepsin	20	34 500	3	1.00 ± 0.06 (5)	1.55
Rhodanese	20	32 900	1*	0.97 ± 0.05 (3)	1.70
Ribonuclease A	5	13 700	4	2.02 ± 0.04 (8)	3.5
Ribonuclease S	5	13 700	4	3.1 ± 0.1 (4)	1.85, 0.79 ($\bar{n} = 2.0$)
Ribonuclease S-protein	5	11 500	4	2.9 ± 0.1 (4)	2.1, 0.86 ($\bar{n} = 2.1$)
Superoxide dismutase†	30	60 000	2	< 0.1 (3)	–
Trypsinogen	5	24 000	6	2.2 ± 0.2 (5)	3.5
Urease	40	489 000	8	6.3 ± 0.5 (3)	3.2

* May be a persulphide group.
† Apoprotein.

2.5 h at 37°C, and then an aliquot was immediately transferred to deaerated 0.2 M-NaOH in the c.s.v. cell. There was no significant change in the $\bar{n}$ values for albumin, chymotrypsin, lysozyme, oxytocin, papain, pepsin and ribonuclease S, but the $\bar{n}$ value for ribonuclease A increased from 2.0 to 2.8 after denaturation, and that for insulin from 0.98 to 2.03. In addition, the rate constants (k_1) for the dissociation of the S–S bonds were substantially greater after denaturation for chymotrypsin, papain, pepsin, and the first two bonds of ribonuclease S.

In Table 2 the c.s.v. diffusion currents (i_d), i.e. the current flowing through the cell during deposition of sulphide, are compared with the stripping peak currents (i_p) for some sulphur-containing species. The ratio of i_d to i_p is characteristic of the diffusing species (eqns. 1 and 2), since the diffusion current is given by:

$$i_d = nFDAc/\delta \qquad (3)$$

where n is the number of electrons involved in the electrode reaction, F is the Faraday, A is the area of the electrode, c and D the bulk concentration and diffusion coefficient, respectively, of the diffusing species, and δ is the thickness of the diffusion layer. The peak current (i_p), on the other hand, is proportional to the quantity of material deposited on

the electrode (Brainina, 1971). By using eqn. (3) and the published values (Stricks & Kolthoff, 1952) for the diffusion coefficients of cysteine (7.0×10^{-6} cm$^2 \cdot$s^{-1}) and Pb^{2+} (8.67×10^{-6} cm$^2 \cdot$s^{-1}), comparison of the diffusion currents (Florence, 1979a) gave a diffusion coefficient of $(1.35 \pm 0.07) \times 10^{-5}$ cm$^2 \cdot$s^{-1} for HS^- in 0.2 M-NaOH at 25°C.

The c.s.v. i_d/i_p values for several proteins and for the ultrafiltrate from an alkaline digest of bovine serum albumin were found to be identical to the value for HS^- (Table 2), whereas elemental sulphur and cysteine persulphide gave much lower values. The low values of i_d and i_p for elemental sulphur probably result from a combination of the reactions:

$$S + Hg \rightarrow HgS \qquad (4)$$

$$4S + 4OH^- \rightleftharpoons 2HS^- + S_2O_3{}^{2-} + H_2O \qquad (5)$$
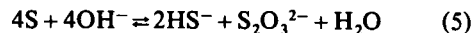
Reaction (4) would provide a stripping current but no diffusion current. In the diffusion layer, however, reaction (5) would be forced to the right by removal of HS^- as HgS, and a diffusion current would result, although its value would be less than that for an equivalent concentration of sulphide.

The oxidation of cysteine persulphide at a mercury electrode may be represented by:

Table 2. *Comparison of c.s.v. deposition currents* (i_d) *and stripping currents* (i_p) *for various sulphur compounds in 0.2 M-NaOH at 25°C*

The deposition currents were measured at -0.35 V versus saturated calomel electrode, and the stripping currents at -0.79 V versus saturated calomel electrode after 3 min deposition. Currents are in μA, and concentrations (c) are mol/l. The i_d/i_p results show means $\pm$ relative s.D. and the number of separate determinations (in parentheses). The protein result was obtained from measurements on bovine serum albumin, chymotrypsin, insulin and trypsinogen. The i_d/i_p ratio for protein was constant throughout the digestion in 0.2 M-NaOH. The bovine serum albumin ultrafiltrate was obtained by filtering through a Diaflo PM30 membrane (N_2 pressure) a solution of albumin that had stood in 0.2 M-NaOH at 25°C for 4 h. The elemental sulphur was added as a solution in pyridine, and cysteine persulphide was prepared by mixing equimolar concentrations of cysteine and sulphur (in pyridine). All solutions contained 10 μg of gelatine/ml.

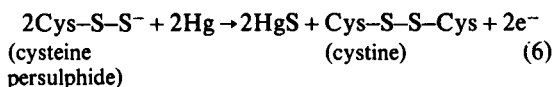| Measured species	$10^{-6} \times i_d/c$ (μA·l·mol^{-1})	$10^{-9} \times i_p/c$ (μA·l·mol^{-1})	$10^3 \times i_d/i_p$
HS$^-$	2.8	1.21	2.3 ± 0.2 (5)
S	0.52	0.50	1.0 ± 0.1 (3)
Cysteine	0.72	0	–
Cysteine persulphide	0.70	0.55	1.3 ± 0.1 (4)
Protein digest	–	–	2.2 ± 0.2 (20)
Albumin ultrafiltrate	–	–	2.3 (1)

Table 3. *Determination of the rate constants* (k_2) *and equilibrium constants* (K_e) *for the dissociation of the intermediate involved in the cleavage of protein S–S bonds in 0.2 M-NaOH at 25°C*

The reaction involved is:

$$A \xrightarrow{k_1} B \underset{k_2}{\overset{k_2}{\rightleftharpoons}} C + HS^-; \quad K_e = [HS^-]^2/(\bar{n}A_0 - [HS^-])$$
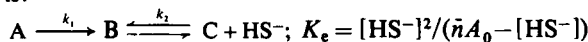
where A is a protein and B is the intermediate. The ratio equilibrium $[HS^-]$ (measured by sulphide-ion-selective electrode)/total $[HS^-]$ was calculated on the basis of one molecule of HS$^-$ liberated/S–S bond dissociated. The constants for chymotrypsinogen were not calculated because of the double value for k_1 (Table 1).

Protein	$10^3 \times k_2$ (min^{-1})	$10^5 \times K_e$ (mol·l^{-1})	$[HS^-]_{equil.}/[HS^-]_{total}$
Albumin, bovine	0.90	3.8	0.41 ($\bar{n} = 5.0$)
α-Chymotrypsin A	2.9	6.9	0.57 ($\bar{n} = 1.0$)
α-Chymotrypsinogen A	–	–	0.24 ($\bar{n} = 2.0$)
Insulin	5.9	11.0	0.56 ($\bar{n} = 1.0$)
Papain	1.4	0.10	0.13 ($\bar{n} = 1.0$)
Trypsinogen	5.4	29	0.69 ($\bar{n} = 2.0$)

$$2Cys-S-S^- + 2Hg \rightarrow 2HgS + Cys-S-S-Cys + 2e^-$$
(cysteine   (cystine)   (6)
persulphide)

Since this reaction involves only one electron/molecule of c.s.v.-active sulphur, whereas the oxidation of HS$^-$ involves two electrons (eqn. 1), and noting that the diffusion coefficient of HS$^-$ is approx. twice that of cysteine, the diffusion current for HS$^-$ should be about four times that of cysteine persulphide, and its peak current about twice. The fact that the experimental results (Table 2) are in good agreement with these calculations lends support to the validity of eqn. (6).

Some non-protein disulphides and other sulphur compounds were studied by c.s.v. in 0.2 M-NaOH. Even at relatively high concentrations, lanthionine, cystine, cystaminium chloride, lipoic acid, dithiodipropionic acid, 2,2'-dithiosalicylic acid, 6,6'-

dithiodinicotinic acid, and 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) gave no trace of a c.s.v. wave after 4 h at 25°C. Dithiodiglycolic acid gave a well-defined sulphide wave with an $\bar{n}$ value of 1.05, and a first-order rate constant, k_1, of 17.1×10^{-2} min^{-1}.

Ion-selective-electrode potentiometry

For all proteins studied, the equilibrium concentration of HS$^-$ measured by the sulphide-ion-selective electrode was always less than that given by c.s.v. (Table 3), and the ion-selective-electrode readings took much longer to reach a constant value than did the c.s.v. peak currents (Fig. 2). The shape of the HS$^-$–time curve (Fig. 2) is typical of a kinetic process involving an induction period and dissociation of an intermediate, i.e. A→B→C (Frost & Pearson, 1961). Comparison of the c.s.v. and ion-selective-electrode results, and the fact that
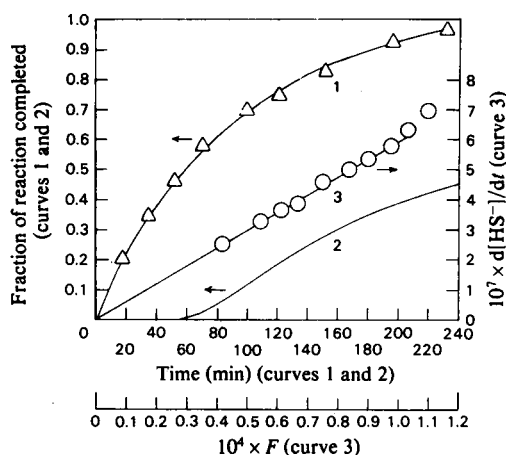
Fig. 2. *Kinetics of the dissociation of an S–S bond of insulin in 0.2 M-NaOH*

Curve 1, increase with time of the c.s.v. wave for 4.52×10^{-7} M-insulin in deaerated 0.2 M-NaOH at 25°C, assuming one S–S bond dissociated to yield one molecule of c.s.v.-active substance; curve 2, increase with time of the concentration of HS$^-$ (measured by a sulphide-ion-selective electrode) in a solution of 1.56×10^{-4} M-insulin in deaerated 0.2 M-NaOH at 25°C, assuming one S–S bond dissociated to yield one molecule of HS$^-$; curve 3, plot of F [the expression inside braces in eqn. (9)] versus the rate of change of HS$^-$ concentration (d[HS$^-$]/dt) as measured by a sulphide-ion-selective electrode (curve 2).

additions of OH$^-$ and HS$^-$ decrease and increase, respectively, the absorbance at 335 nm of a protein solution at equilibrium in 0.2 M-NaOH, indicates that the dissociation of the intermediate does not go to completion, but is involved in a mobile equilibrium with HS$^-$:

$$A \xrightarrow{k_1} B \underset{}{\overset{k_2}{\rightleftarrows}} C + HS^- \qquad (7)$$

where A is a protein and B is the intermediate compound, perhaps a persulphide. The ion-selective electrode potentiometric measurements would not disturb this equilibrium, but the c.s.v. process would lead to rapid dissociation of the intermediate in the diffusion layer as a result of the removal of HS$^-$ as HgS. The equilibrium constant for the dissociation of B is given by:

$$K_e = [HS^-]^2/(\bar{n}A_0 - [HS^-]) \qquad (8)$$

where A_0 is the initial protein concentration, and $\bar{n}$ the number of disulphide bonds dissociated. A rate equation was derived for eqn. (7):

where [HS$^-$]$_e$ is the concentration of HS$^-$ at equilibrium, and k_1 is the first order rate constant measured by c.s.v. (Table 1). A plot of d[HS$^-$]/dt versus the expression (F) inside the braces (eqn. 9) should pass through the origin and have a slope of k_2 (Fig. 2). All the proteins studied by ion-selective electrode showed good agreement with eqn. (9), whereas a very poor correlation was obtained if the results were used in the rate equation for a non-equilibrium reaction, i.e. A→B→C (Frost & Pearson, 1961). The values calculated for k_2 and K_e are collected in Table 3.

Oxidized glutathione, cystine and thiourea gave a release of HS$^-$ too low to be measured by the ion-selective electrode after 2 h at 25°C in 0.2 M-NaOH. The odour of H$_2$S could be detected, however, when an alkaline digest of glutathione was acidified, but the same was not true for cystine (Florence, 1979b). The following compounds did produce measurable HS$^-$ in 0.2 M-NaOH, and the equilibrium concentration of HS$^-$ found is given as a percentage of that expected for $\bar{n} = 1$: elemental sulphur (added as a solution in pyridine), 10.3; cysteine persulphide, 2.10; thioacetamide, 2.91; and dithiodiglycolic acid, 11.8. The plot of HS$^-$ versus time for dithiodiglycolic acid in 0.2 M-NaOH did not show an induction period, presumably because k_1 and k_2 are too large. A value of 1.26×10^{-5} mol·l^{-1} was calculated for K_e, and, by using eqn. (9) and the c.s.v. value of 17.1×10^{-2} min^{-1} for k_1, an approximate value of 0.5 min^{-1} was obtained for k_2.

The assumption has been made in these calculations that the c.s.v. and ion-selective-electrode results can be compared, even though the protein concentrations used in the c.s.v. experiments were often more than two orders of magnitude lower than those used in ion-selective-electrode potentiometry. This difference was unavoidable because of the limited concentration ranges that can be covered by the two techniques. However, it is unlikely that a significant change in reaction mechanism would occur between these concentrations, because both techniques use very dilute protein solutions (less than 1×10^{-4} M) and consistent results were obtained within the concentration ranges used for c.s.v. and ion-selective-electrode potentiometry.

Ultrafiltration experiments

Ultrafiltration experiments were carried out on digests of bovine serum albumin in 0.2 M-NaOH in an attempt to separate HS$^-$ from high-molecular-weight persulphides or other sulphur-labile compounds. A 1×10^{-4} M-solution of albumin that had

$$\frac{d[HS^-]}{dt} = k_2 \left\{ \bar{n}A_0 \left[(1 - e^{-k_1 t}) - \left(\frac{[HS^-]}{[HS^-]_e} \right)^2 \right] - [HS^-] \left(1 - \frac{[HS^-]}{[HS^-]_e} \right) \right\} \qquad (9)$$

stood in deaerated 0.2 M-NaOH at 25°C for 4 h was ultrafiltrated, and an aliquot of the filtrate was used for c.s.v. analysis. The c.s.v. sulphide wave appeared immediately and did not increase with time. The i_d/i_p value was identical to that for HS⁻ (Table 2), and the height of the wave corresponded to an $\bar{n}$ value of 5.3, which is similar to the value obtained by direct c.s.v. (Table 1). Other workers (Catsimpoolas & Wood, 1964; Cecil & Weitzman, 1964) have reported that five S–S bonds in bovine serum albumin can be reduced. Analysis of the ultrafiltrate with the ion-selective electrode showed that 86% of the expected ($\bar{n} = 5.0$) HS⁻ was present, which can be compared with the 41% of theoretical HS⁻ found for bovine serum albumin at equilibrium in 0.2 M-NaOH (Table 3).

The ultrafiltrate was also analysed spectrofluorimetrically by using fluorescein mercuric acetate. In 0.2 M-NaOH it was found that HS⁻, thiols and disulphides gave equal depression of the fluorescence for the same molar concentration of sulphur. In a Tris/HCl buffer of pH 7.5, however, disulphides had no effect on the fluorescence, but HS⁻ and –SH reacted with equal sensitivity. Presumably persulphides would also react with fluorescein mercuric acetate at pH 7.5.

The albumin ultrafiltrate was analysed at pH 7.5 before and after acidification and purging with N_2 to remove H_2S. Before removal of H_2S the depression of fluorescence corresponded to an $\bar{n}$ value of 5.2, and after removal to only 0.3, indicating that few fluorescein mercuric acetate-active sulphur compounds other than HS⁻ were present in the ultrafiltrate. The same experiment was carried out on the unfiltered albumin digest. The total concentration of fluorescein mercuric acetate-active compounds at pH 7.5 corresponded to $\bar{n} = 9.5$ and, after acidification and purging, to 5.0. It is apparent that fluorescein mercuric acetate reacts with both sulphur atoms of an alkaline-dissociated S–S bond, and that one of these sulphur atoms can be removed as H_2S. Thiols, persulphides and any other fluorescein mercuric acetate-active sulphur compounds produced by alkaline dissociation of bovine serum albumin are retained by the membrane filter, and only HS⁻ is found in the filtrate.

U.v.-visible spectrophotometry

Wavelength–absorbance curves were recorded for many of the proteins after digestion in 0.2 M-NaOH at 25°C. All proteins with S–S bridges gave an absorbance at 300–350 nm that increased with time. The absorbance at the arbitrarily chosen wavelength of 335 nm reached a maximum after 100–200 min. A difference spectrum for chymotrypsin, obtained by subtracting the spectrum recorded immediately after mixing from that recorded after 47 min is shown in Fig. 3. The difference spectra of
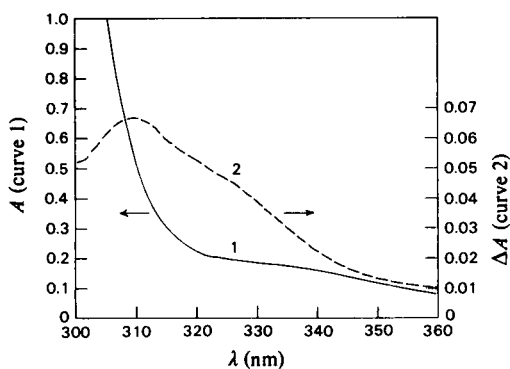

Fig. 3. *Spectra of 1.06×10^{-4} M-chymotrypsin in deaerated 0.2 M-NaOH (1-cm cell)*
Curve 1, after incubation in deaerated 0.2 M-NaOH at 25°C for 3 min, versus 0.2 M-NaOH; curve 2, difference between the spectra recorded after 47 min and after 3 min.

the proteins usually showed absorbance maxima near 310 nm and 235 nm, with molar absorption coefficients (ε) at equilibrium of $(1.0–2.0) \times 10^3$ and $(0.65–1.0) \times 10^4$ litre·mol⁻¹·cm⁻¹ respectively, per S–S bond broken. An exception was oxidized glutathione, which gave an increase in ε of 0.69×10^4 litre·mol⁻¹·cm⁻¹ for $\bar{n} = 1$ at the absorbance maximum of 224 nm, even though its $\bar{n}$ value is only 0.076 (Table 1). Absorbance readings were taken as a function of time at 335 nm and other fixed wavelengths, by using the high-precision manual spectrophotometer. From these results, first-order rate constants were calculated (Table 4). Only data from the first 20 min of the reaction were used in these calculations, so as to avoid any possible complications from reactions between protein S–S bonds and liberated HS⁻ (Cavallini et al., 1970) or from dissociation of a persulphide intermediate. Although the spectrophotometric k_1 values are not as accurate as those obtained by c.s.v., the results were generally in agreement (Tables 1 and 4). The $\bar{n}$ values calculated in Table 4 are only approximate, because the intermediate protein products giving rise to the absorbance at 335 nm would not necessarily dissociate to the same degree, or at the same rate, as the insulin intermediate (Table 3). Nevertheless, in most cases the c.s.v. and spectrophotometric $\bar{n}$ values agree quite well.

Dithiodiglycolic acid in 0.2 M-NaOH gave well defined peaks at 230 nm and 335 nm that increased with time. The spectra for dithiodiglycolic acid, HS⁻, disulphide ion (S_2^{2-}) and cysteine persulphide (the glutathione persulphide spectrum was almost identical) are shown in Fig. 4. Cystine and dithiodipropionic acid gave no increase in absorbance at

Table 4. *Spectrophotometric first-order rate constants for the cleavage of protein S–S bridges in 0.2 M-NaOH at 25°C*
The absorbance of protein solutions in deaerated 0.2 M-NaOH was measured at 335 nm in sealed 1 cm cuvettes as a function of time. Rate constants (k_1) were calculated from the first 20 min of reaction only. The maximum molar absorption coefficient ($\varepsilon_{max.}$) was calculated from the maximum absorbance, corrected for initial absorbance. The $\bar{n}$ values were calculated from $\varepsilon_{max.}$, assuming that insulin has $\bar{n} = 1.00$.

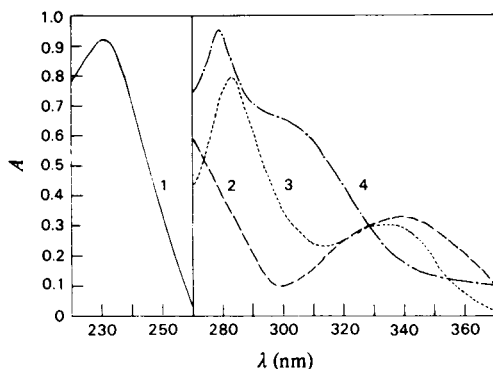| Protein or peptide	$10^2 \times k_1$ (min^{-1})	$\varepsilon_{max.}$ at 335 nm (litre·mol^{-1}·cm^{-1})	No. of S–S bridges cleaved ($\bar{n}$)
Albumin, bovine	2.1	5130	5.50
α-Chymotrypsin A	3.3	905	0.98
Glutathione, oxidized	2.4	153	0.17
Insulin	2.1	925	1.00
Papain	2.8	1220	1.32
Pepsin	1.3	936	1.01
Ribonuclease A	2.3	1712	1.85
Trypsinogen	4.2	1940	2.10

Fig. 4. *Spectra of some sulphur compounds in 0.2 M-NaOH (1-cm cell)*
Curve 1, sulphide (HS$^-$): 1.13×10^{-4} M-HS$^-$ in deaerated 0.2 M-NaOH versus 0.2 M-NaOH; $\lambda_{max.}$ 231 nm, ε 8.15×10^3 litre·mol^{-1}·cm^{-1} at 231 nm. Curve 2, dithiodiglycolic acid: difference spectrum for 1.04×10^{-3} M-dithiodiglycolic acid in deaerated 0.2 M-NaOH; the curve shown is a plot of the absorbance difference between spectra recorded after 104 min and after 3 min; $\lambda_{max.}$ 333 nm. Curve 3, cysteine persulphide (Cys–S–S$^-$): 1.74×10^{-2} M-cysteine + 1.02×10^{-3} M-sulphur (in pyridine) in deaerated 0.2 M-NaOH versus a blank consisting of 0.2 M-NaOH plus the same concentrations of cysteine and pyridine as the sample. Measured immediately; $\lambda_{max.}$ 283 nm and 340 nm, ε 7.84×10^2 and 3.20×10^2 litre·mol^{-1}·cm^{-1}, respectively, based on the sulphur concentration. Curve 4, disulphide (S$_2{}^{2-}$): 4.15×10^{-2} M-HS$^-$ + 6.63×10^{-4} M-sulphur (in pyridine) in deaerated 0.2 M-NaOH versus a blank consisting of 0.2 M-NaOH plus the same concentrations of HS$^-$ and pyridine as in the sample. Measured immediately; $\lambda_{max.}$ 279 nm, ε 1.43×10^3 litre·mol^{-1}·cm^{-1} at 279 nm based on the sulphur concentration.

335 nm, and the compounds lanthionine, mercaptoacetic acid, acrylic acid, glyoxylic acid and thiourea were optically transparent at wavelengths greater than 280 nm. The addition of a large excess of CN$^-$ to a solution of dithiodiglycolic acid in 0.2 M-NaOH had no effect on the absorbance at 335 nm, but CN$^-$ eliminated the absorbance of cysteine persulphide at 335 nm, and substantially decreased the absorbance of the protein solutions at that wavelength.

The reaction between chymotrypsin and HS$^-$ was studied at pH 12 (deaerated 0.01 M-NaOH) by monitoring the absorbance at 335 nm (Cavallini *et al.*, 1970). Previous work (Florence, 1979*b*) showed that k_1 (Table 1) was proportional to OH$^-$ concentration, so negligible reaction should take place between the protein and OH$^-$ at this pH. Using 3.86×10^{-4} M-chymotrypsin and 2.30×10^{-3} M HS$^-$, a second order rate constant of 6.5 litre·mol^{-1}·min^{-1} was calculated from the rate plot, which is similar to the value of 17 litre·mol^{-1}·min^{-1} that can be derived from the data of Cavallini *et al.* (1970) for the reaction between insulin and HS$^-$. These reactions are too slow to be significant at the protein concentrations used in the c.s.v. and ion-selective-electrode experiments, but could have some effect at the later stages of the spectrophotometric measurements. The difference spectra for chymotrypsin plus HS$^-$ had absorbance maxima at 310 nm, the same wavelength at which a maximum occurred for solutions of chymotrypsin digested in 0.2 M-NaOH (Fig. 3).

No elemental sulphur was detected by n-heptane extraction at pH 8 of 0.2 M-NaOH digests of bovine serum albumin, chymotrypsin, insulin, lysozyme or trypsinogen. All these proteins gave results equivalent to less than 0.1 molecule of elemental sulphur/S–S bond dissociated.

Discussion

It was shown earlier (Florence, 1979*b*) that proteins do not appear to suffer peptide-chain scission in dilute alkali at room temperature, and that the S–S bonds degraded were probably those most exposed, or solvent accessible (Bohak, 1964). This present work shows some unexpected differ-

ences in the c.s.v. behaviour in 0.2 M-NaOH of proteins with very similar structures.

Chymotrypsin has one, and chymotrypsinogen and trypsinogen have two, S–S bond(s) broken in 0.2 M-NaOH (Table 1). Reduction of a single bond (Cys$_{191}$–Cys$_{220}$) in chymotrypsin by dithiothreitol is known to cause inactivation of the enzyme (Stanton & Viswanatha, 1971; Martin & Viswanatha, 1975). The Cys$_{191}$–Cys$_{220}$ S–S bond in chymotrypsinogen can also be reduced, although the rate of reduction is very small (Sondack & Light, 1971). By contrast, the homologous bond in trypsinogen (Cys$_{179}$–Cys$_{203}$) is readily reduced, followed by the Cys$_{122}$–Cys$_{189}$ S–S bond, which requires a higher concentration of reductant (Sondack & Light, 1971). Similar results were found by degradation in 0.2 M-NaOH; the labile S–S bonds of chymotrypsin and trypsinogen are rapidly attacked, but the first bond of chymotrypsinogen is only slowly dissociated (Table 1). The double slope rate plot for chymotrypsinogen, intersecting at $\bar{n} = 1$, suggests that destruction of the first S–S bond is rate determining, and that it must be opened before the second bond can be attacked.

Ribonuclease A with two of its four S–S bonds reduced is a unique molecular species. Loss of the Cys$_{65}$–Cys$_{72}$ and Cys$_{58}$–Cys$_{100}$ S–S bonds leaves the conformation practically unaffected, and the enzyme retains full biological activity (Neumann et al., 1967; Sperling et al., 1969; Tamburro et al., 1970). Ribonuclease S is a subtilisin-produced derivative of ribonuclease A, where the peptide chain is broken between alanine-20 and serine-21. The S-protein and S-peptide are held together by powerful non-covalent bonds, and ribonucleases A and S show very similar chemical and biological behaviour (Sherwood & Potts, 1965; Woodfin & Massey, 1968). Despite this similarity, several studies have shown that ribonuclease S is not as compact or as tightly folded as the native enzyme, and that it has less conformational stability (Sherwood & Potts, 1965). The differences observed in 0.2 M-NaOH (Table 1) are quite striking. An extra S–S bond is broken in ribonuclease S and S-protein, although the first two bonds are dissociated at a slower rate than those of ribonuclease A. Ribonuclease S and S-protein have almost identical c.s.v. characteristics, which would be expected because the ionic bonds binding the peptide to the protein would probably dissociate in the alkaline medium. Majdrakov & Krysteva-Majdrakova (1974) suggested that the scission of the peptide chain between residues 20 and 21 in ribonuclease A may cause increased exposure of the Cys$_{40}$–Cys$_{95}$ bond. Heating with 6 M-guanidine hydrochloride before alkaline degradation allowed an additional S–S bond to be dissociated in ribonuclease A, and the three bonds all reacted at the same rate ($k_1 = 3.3$ min^{-1}).

The two S–S bonds of deoxyribonuclease are reduced with unusual ease by mercaptoethanol at room temperature, but if Ca^{2+} (4 mM) is present during reduction only one S–S bond is reduced (Price et al., 1969). The loss of only one S–S bond (Cys$_{98}$–Cys$_{101}$) does not affect catalytic activity, but loss of the essential second bond (Cys$_{170}$–Cys$_{206}$) gives an inactive product (Liao et al., 1973). Despite the ease of reduction of deoxyribonuclease, alkaline digestion leads to the dissociation of only one S–S bond (Table 1). Perhaps the high concentration of Na$^+$ (0.2 M) acts like Ca^{2+} to protect the second bond.

Urease contains eight S–S bonds, all of which are reducible in 8 M-urea (Staples & Reithel, 1976). Six of these bonds are attacked in 0.2 M-NaOH. Two of the four S–S bonds of lipoamide dehydrogenase (the two interchain S–S bonds) can be reduced by dithionite (Massey et al., 1962), which is the same number dissociated as in 0.2 M-NaOH. In this medium the first S–S bond reacts much faster than the second (Table 1). The S–S bonds of lysozyme are particularly resistant to reduction; even in 8 M-urea, reaction with dithiothreitol or sulphite is sluggish and only two or three of the total of four bonds are reduced (Azari, 1966; Iyer & Klee, 1973). In 0.2 M-NaOH three S–S bonds are dissociated, but attack on the first bond is extremely slow. After this bond is broken, however, the next two bonds dissociate at a reasonably fast rate. The additional bond attacked in insulin when a preliminary treatment with guanidine hydrochloride was used is probably the second interchain S–S bond (Cys$_{A20}$–Cys$_{B19}$), because the single intrachain bond (Cys$_{A6}$–Cys$_{A11}$) is buried in a hydrophobic pocket (Blundell et al., 1972).
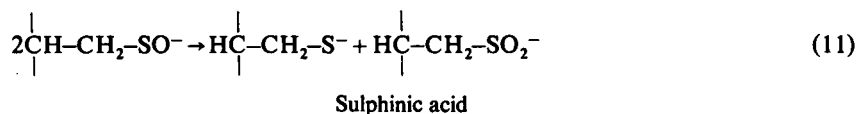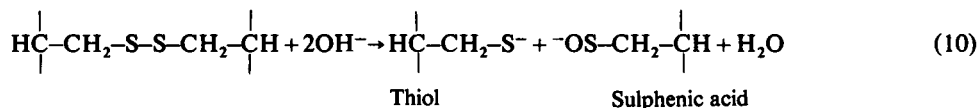
There is some dispute about the existence of an S–S bond in bovine rhodanese. Earlier workers (Wang & Volini, 1968; Blumenthal & Heinrikson, 1971) claimed that rhodanese consisted of two identical subunits, with a single intermolecular S–S bond, plus two free thiol groups. But in a recent study Ploegman et al. (1978) showed that rhodanese is a single polypeptide chain and contains no S–S bonds. There are four cysteine residues, and one of these, Cys-247, is at the active centre and forms a persulphide bond with the substrate sulphur. This persulphide group is stabilized by hydrogen bonds from nearby amino acids. In 0.2 M-NaOH, however, the sample of bovine rhodanese available behaved like a typical S–S bond-containing protein, the sulphide wave increasing with time at a rate similar to other proteins (Table 1). If a persulphide, rather than an S–S, group were present, the full wave height may be expected to appear immediately and remain constant. It is possible that some unfolding of the enzyme has to take place before the persulphide group is available for reaction at the electrode.

Bovine superoxide dismutase consists of two identical subunits, each having a single intra-molecular S–S bond. These bonds are critical to protein stability and subunit cohesion (Abernethy *et al.*, 1974). The enzyme is unusually stable towards denaturants and organic solvents; most of its activity is retained in 8 M-urea or 86% ethanol (Forman &
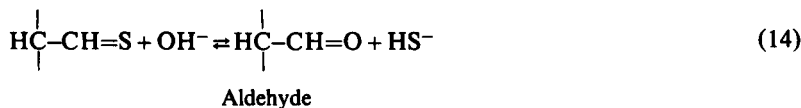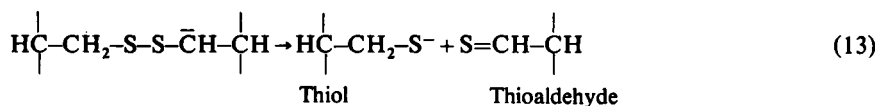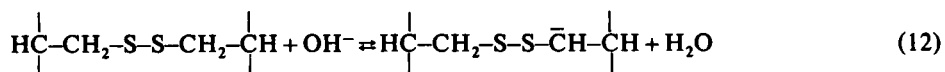
reaction involved in the dissociation of the S–S bonds of these two compounds is obviously different from that of the other proteins.

Three distinct mechanisms have been proposed for the alkaline degradation of S–S bonds in proteins and other compounds: hydrolysis, α-elimination and β-elimination (Danehy, 1966; Nashef *et al.*, 1977a).

Hydrolysis:
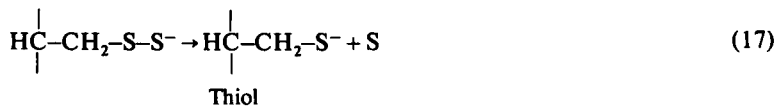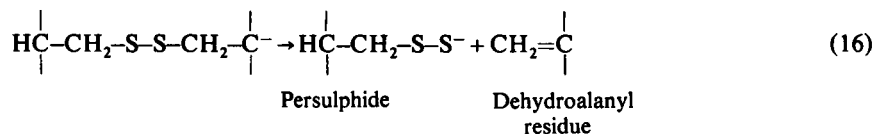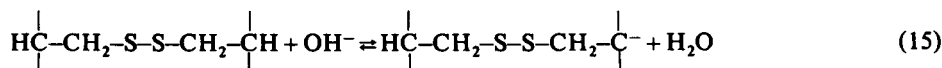
$$HC–CH_2–S–S–CH_2–CH + 2OH^- \rightarrow HC–CH_2–S^- + {}^-OS–CH_2–CH + H_2O \tag{10}$$

               Thiol   Sulphenic acid

$$2CH–CH_2–SO^- \rightarrow HC–CH_2–S^- + HC–CH_2–SO_2^- \tag{11}$$

               Sulphinic acid

α-Elimination:

$$HC–CH_2–S–S–CH_2–CH + OH^- \rightleftharpoons HC–CH_2–S–S–\bar{C}H–CH + H_2O \tag{12}$$

$$HC–CH_2–S–S–\bar{C}H–CH \rightarrow HC–CH_2–S^- + S=CH–CH \tag{13}$$

            Thiol   Thioaldehyde

$$HC–CH=S + OH^- \rightleftharpoons HC–CH=O + HS^- \tag{14}$$

            Aldehyde

β-Elimination

$$HC–CH_2–S–S–CH_2–CH + OH^- \rightleftharpoons HC–CH_2–S–S–CH_2–\bar{C}^- + H_2O \tag{15}$$

$$HC–CH_2–S–S–CH_2–\bar{C}^- \rightarrow HC–CH_2–S–S^- + CH_2=C \tag{16}$$

        Persulphide   Dehydroalanyl residue

$$HC–CH_2–S–S^- \rightarrow HC–CH_2–S^- + S \tag{17}$$

           Thiol

Fridovich, 1973). Neither the native nor the apo-enzyme showed any significant dissociation of the S–S bonds in 0.2 M-NaOH. The resistance of these bonds to chemical attack is doubtlessly essential to its efficient functioning as a free radical scavenger.

The small polypeptides oxytocin and oxidized glutathione yielded similar and low $\bar{n}$ values, even though the reactions appeared to go to completion (Table 1). The increase in absorbance at 335 nm also indicated a low concentration of persulphide (Table 4), but the increase at 224 nm for glutathione was close to that expected for $\bar{n} = 1$. The predominant
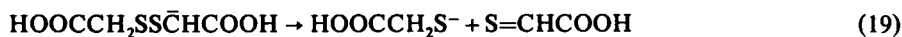
Hydrolysis reaction

Danehy & Parameswaran (1968) believe that the hydrolysis reaction, i.e. the direct nucleophilic attack of OH^- on the S–S bridge, will take place unless attack is inhibited by a negative charge near the S–S bond or if there is a sufficiently labile proton bonded to an α- or β-carbon atom, in which case elimination reactions can take place. The reactivity of OH^- towards the S–S bond is quite weak compared with other nucleophilic reagents, the order being $HS^- > RS^- > CN^- > SO_3^{2-} > OH^-$, so it is often the basicity of OH^- rather than its nucleo-
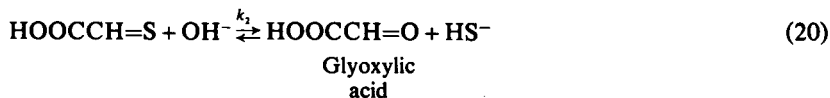
philic character that is important (Roy & Trudinger, 1970). Ellman's reagent, which is an aromatic disulphide and contains no eliminatable hydrogens, was found in this study to give no trace of HS^-. It apparently decomposes by a very rapid hydrolysis reaction to give a thiol and a sulphinic acid (Danehy et al., 1971; Nashef et al., 1977a). Danehy & Hunter (1967) found that HS^- is not a product of the alkaline decomposition of cystine at 35°C, the only products being cysteine and cysteic acid formed by oxidation of the sulphinic acid. Cystine therefore decomposes by a hydrolysis reaction, which explains the absence of a c.s.v. wave (Florence, 1979b). Apparently the free amino group of cystine neutralizes the effect of the COO^- group and allows direct attack by OH^- on the S–S bond. If, however, the amino group is acetylated, or the carboxyl group esterified, the acidity of the β-hydrogen is increased and the modified cystine decomposes by β-elimination (Schneider & Westley, 1969). Cecil & McPhee (1959) pointed out that when cystine is incorporated in peptide chains, the β-carbon has two electron-attracting groups attached (–CONH– and –NHCO–), so its proton is very labile and elimination reactions are greatly facilitated.

α-Elimination reaction

The classical α-elimination reaction, (eqns. 12–14) (Rosenthal & Oster, 1961; Kornblum & De La Mare, 1951), may occur only rarely, if at all, in polypeptides. The most established example of a disulphide that undergoes alkaline decomposition by α-elimination is dithiodiglycolic acid, $HOOCCH_2SSCH_2COOH$ (Rosenthal & Oster, 1961):

NaOH (Fig. 4) was unaffected by CN^-, which would be expected because CN^- does not react with $-C=S$. An α-elimination reaction for dithiodiglycolic acid is especially favoured, because the two ionized carboxyl groups adjacent to the S–S bond would inhibit direct hydrolysis, a β-elimination reaction cannot occur (there is no β-hydrogen), and the thiol form ($HS–C=$) which usually predominates over the thione tautomer ($S=C–$) in thiopyruvic acids (Campaigne & Cline, 1956) cannot exist in thioglyoxylic acid. Apparently the ionized carboxyl groups do not prevent the acid-base reaction between OH^- and the α-hydrogen.

The degradation of proteins in alkali is unlikely to involve an α-elimination because (a) with all proteins studied, CN^- eliminates or substantially diminishes the absorbance at 335 nm; (b) the mechanism does not explain the formation of dehydroalanine residues, and hence lanthionine, lysinoalanine and other dehydroalanine reaction products found in alkali-treated proteins; and (c) aldehyde groups have never been found in protein digests (Stapleton & Swan, 1960).
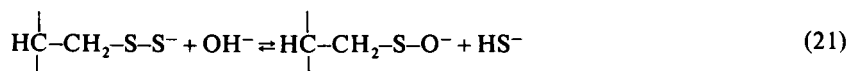
β-Elimination reaction

The results of this study strongly point to elimination of a β-hydrogen and formation of a persulphide intermediate (eqns. 15–17) as being the first steps in the degradation of protein disulphide bonds by dilute alkali at 25°C. The evidence for β-elimination includes (a) the efficient formation of lanthionine, lysinoalanine and other dehydroalanine reaction products; (b) the production of an absorbance in the 300–350 nm region which increases with time at a rate similar to the rate of growth of the

$$HOOCCH_2SSCH_2COOH + OH^- \rightleftharpoons HOOCCH_2SS\bar{C}HCOOH + H_2O \qquad (18)$$

$$HOOCCH_2SS\bar{C}HCOOH \rightarrow \underset{\substack{\text{Mercaptoacetic}\\\text{acid}}}{HOOCCH_2S^-} + \underset{\substack{\text{Thioglyoxylic}\\\text{acid}}}{S=CHCOOH} \qquad (19)$$

$$HOOCCH=S + OH^- \overset{k_2}{\underset{}{\rightleftharpoons}} \underset{\substack{\text{Glyoxylic}\\\text{acid}}}{HOOCCH=O} + HS^- \qquad (20)$$

Thioglyoxylic acid is known to be intensely yellow (it absorbs at 335 nm) and is in equilibrium in aqueous solution with HS^- (Rosenthal & Oster, 1961). In the present study a value of 1.26×10^{-5} mol·l⁻¹ was calculated for the equilibrium constant, K_e, of eqn. (20), the equilibrium being established rapidly ($k_2 = 0.5$ min⁻¹) compared with the proteins. Dithiodiglycolic acid in 0.2 M-NaOH had a c.s.v. value close to 1, which would result from reaction (20) being forced to the right by formation of mercuric sulphide. The absorbance peak at 335 nm for dithiodiglycolic acid in 0.2 M-

c.s.v. wave, and which can be eliminated or diminished by the addition of CN^- to form thiocyanate; and (c) the similarity of the increase in absorbance in the 300–350 nm region of proteins treated with alkali and with HS^-. The nucleophilic attack of HS^- on S–S bonds is known to give persulphide as a primary product (Rao & Gorin, 1959; Cavallini et al., 1970), and in this study it was shown that an absorbance peak occurred at 310 nm when chymotrypsin was treated with either HS^- or 0.2 M-NaOH.

A problem with the classical β-elimination scheme

is that sulphur, rather than HS^-, is supposed to be the decomposition product of persulphide. No free sulphur was found in the alkaline digests of several proteins, in agreement with earlier findings (Danehy, 1966; Catsimpoolas & Wood, 1964; Gawron & Odstrchel, 1967). The detection of sulphur in alkaline digests of lysozyme (Nashef et al., 1977a) may have been due to the use of an inappropriate analytical method. Another analytical problem has often occurred in the determination of thiol. Many workers studying the effects of alkali on proteins used Ellman's reagent at pH 8 as a specific reagent for the direct determination of thiol groups in the protein digests, without appreciating that this reagent is actually twice as sensitive towards sulphide (and, presumably, persulphide) as it is for thiol groups (Benedict & Stedman, 1970; Nashef et al., 1977b). Fluorescein mercuric acetate also reacts with both HS^- and $-SH$, and it was shown here that

present study, found that there was an induction period for the appearance of HS^- in insulin solutions incubated in 0.5 M-NaOH at 37°C, and that the equilibrium concentration of HS^- was only about 40% of the theoretical persulphide concentration. The rate-controlling step for the production of persulphide must be proton abstraction from the β-carbon atom (eqn. 15), because the first-order rate constants for both the growth of the c.s.v. wave and the absorbance at 335 nm are directly proportional to OH^- concentration (Florence, 1979b) and the rate of alkaline hydrolysis increased with increasing ionic strength (Donovan & White, 1971; Florence, 1979b), the result expected for reaction of a negative ion (OH^-) with a negatively-charged protein.

The mode of decomposition of a persulphide (eqn. 7) which best fits the experimental evidence is hydrolysis to form a sulphenic acid (Cavallini et al., 1960):

$$HC{-}CH_2{-}S{-}S^- + OH^- \rightleftharpoons HC{-}CH_2{-}S{-}O^- + HS^- \tag{21}$$

a solution of bovine serum albumin in 0.2 M-NaOH produced approx. equimolar concentrations of sulphide and another fluorescein mercuric acetate-active substance, possibly thiol.

Strong evidence was accumulated to show that, in solutions of proteins in 0.2 M-NaOH, free HS^- is in mobile equilibrium with an intermediate, probably a persulphide. Comparison of the c.s.v. diffusion and stripping currents showed unequivocally that HS^-, and not S_2^{2-} or a persulphide, is the diffusing species. Proteins typically have diffusion coefficients in the range 0.5×10^6–1.0×10^{-6} cm$^2 \cdot$ s^{-1} at 25°C (Cecil & Weitzman, 1964), which can be compared with the diffusion coefficient of $(1.35 \pm 0.07) \times 10^{-5}$ cm$^2 \cdot$ s^{-1} determined here for HS^-. Protein persulphide therefore must not diffuse directly to the electrode, because the diffusion currents observed are too large. Instead, the persulphide must

The sulphenic acid would then react further to produce thiol and a sulphinic acid (eqn. 11).

The dissociations of oxidized glutathione (GSSG) and oxytocin obviously do not follow the normal β-elimination path, since only about 10% of the concentration of persulphide expected for $\bar{n} = 1$ is released (Tables 1 and 4), even though c.s.v. peak heights and the absorbance at 224 nm for GSSG indicated that the reaction had gone to completion. Asquith & Carthew (1972) and Moorehead (1976) showed that dehydroalanine residues (which absorb near 230 nm) are stoichiometrically formed from GSSG in alkali, an observation that supports a β-elimination (eqn. 16). Moorehead (1976) proposed that alkaline dissociation of GSSG gives an episulphide, rather than a persulphide, which then decomposes to a dehydroalanine residue and sulphur:

$$HC{-}CH_2{-}S{-}S{-}CH_2{-}CH + OH^- \rightarrow HC{-}CH_2{-}S^- + C{-}CH_2 + H_2O \tag{22}$$

Thiol   Episulphide
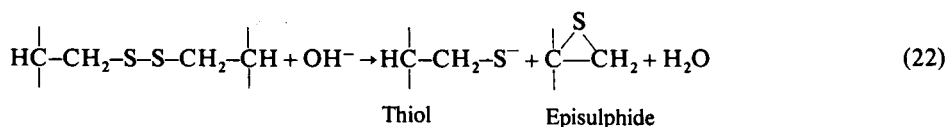
decompose almost quantitatively in the diffusion layer to liberate an equivalent of HS^-. This behaviour is analogous to the reduction of some metal–organic complexes at a mercury electrode (Heyrovský & Kůta, 1966). The persulphide also liberates most of its HS^- under the concentration gradient existing at the surface of an ultrafiltration membrane, a further indication of its lability. Schneider & Westley (1969), in agreement with the

$$C{-}CH_2 \rightarrow C{=}CH_2 + S \tag{23}$$

Dehydroalanine
residue
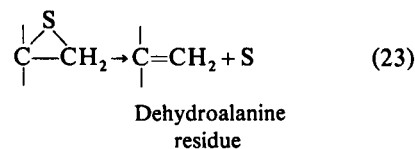
Episulphide formation is also believed to be involved in the alkaline dissociation of dithiodipro-

pionic acid (Danehy & Hunter, 1967). This acid gave no c.s.v. wave, and no absorbance at 335 nm.

Some aspects of the alkaline degradation of protein S–S bonds remain unclear. If the primary reaction product is indeed a persulphide, then its c.s.v. behaviour and its spectrum between 300 and 350 nm vary significantly from that of cysteine persulphide. It is not surprising that a persulphide group attached to a large peptide chain does not enter into the same electrode reactions as the simple cysteine derivative, which has a much higher diffusion coefficient and can be oxidized at the electrode surface to cystine (eqn. 6). The difference between the spectra of the two types of persulphides (Figs. 3 and 4) may be a result of the two electron-attracting peptide bonds causing a 25 nm hypsochromic shift with respect to free cysteine (Campaigne & Cline, 1956). Perhaps protein persulphide forms a ring compound by accepting hydrogen bonds from neighbouring amino acids (Ploegman et al., 1978) and this also alters its properties by comparison with the cysteine derivative.

It is more difficult to explain why GSSG and oxytocin should behave so completely differently from both cystine and the proteins. The distribution of charge in the vicinity of the S–S bonds may be quite different for proteins and small polypeptides, and this could influence the mode of decomposition. The answer to this question will require more detailed analyses of the products of alkaline decomposition, and the study of polypeptides with molecular weights between those of oxytocin and insulin.

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