Generation of an Acid-Stable and Protein-Bound Persulfide-like Residue in Alkali- or Sulfhydryl-Treated Insulin by a Mechanism Consonant with the β-Elimination Hypothesis of Disulfide Bond Lysis†

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ABSTRACT: The mechanism of alkali-disulfide bond lysis in proteins is unclear. According to the β-elimination mechanism, persulfide (RSSH) and dehydroalanine ($RCH{=}CH_2$) are generated whereas thiol (RSH) and sulfinic acid (RSO_2H) are the products of the alternate alkaline hydrolysis mechanism. Insulin is a small protein consisting of two polypeptides containing one intrachain and two interchain disulfide bonds. We conclude that the lysis of the disulfide bonds of insulin in alkali (pH 13, 22 °C) or by sulfhydryl compounds (pH 9, 22 °C) proceeds by the β-elimination mechanism for the following reasons: (1) For each disulfide bond lysed, one hydroxide ion was consumed and one sulfhydryl was generated as predicted by the β-elimination mechanism. (2) The intensity of a chromophore generated at 240 nm (extinction coefficient = 16 000 M^{-1} cm^{-1}) is consistent with the generation of persulfide and dehydroalanine and greater than could be accounted for by sulfinic acid and thiol. (3) Simple model persulfides are reported to absorb weakly at 335 nm. A chromophore, reported by others as a broad shoulder on the tyrosyl absorption band, was estimated from first derivative difference spectra to be centered at 323 nm (extinction coefficient = 670 M^{-1} cm^{-1}). (4) The chromophore at 323 nm was abolished by cyanide with the formation of thiocyanate. Two moles of sulfhydryl was generated per mole of insulin and at least 1.5 mol was cyanolyzable. (5) Half a mole of the cyanolyzable sulfur was acid labile and formed hydrogen sulfide and ligation products as predicted ($2RSS^- + 2H^+ \rightleftharpoons R{-}S{-}S{-}R + H_2S + S$). Contrary to the predicted reactivity of persulfide in acid, 1 mol of cyanolyzable sulfur was stable per mol of acidified insulin. This acid-stable and cyanolyzable sulfur residue eluted with the protein fraction on Sephadex G-25. This persulfide was also generated by thiolate ions. The alkali-treated insulin was cleaved into smaller components by lysis of the two interchain disulfide bonds. The intrachain disulfide bond was unreactive in alkali except under denaturing conditions.

Two mechanisms are commonly invoked to explain the alkaline fission of disulfide bonds in proteins or low molecular weight model compounds. The alkaline hydrolysis mechanism involves the direct attack of hydroxide ion on a sulfur atom, and by a subsequent sulfur–sulfur split, thiol (RSH) and sulfinic acid (RSO_2H) are generated. The β-elimination mechanism postulates that a hydroxide ion removes a proton from the α carbon (β to a sulfur atom) and that this results in carbon–sulfur fission with the concomitant generation of persulfide ion (RSS^-) and dehydroalanine ($R_2C{=}CH_2$). Several studies have been reported which support one or the other of these mechanisms [Schneider & Westley, 1969; Donovan & White, 1971; for reviews, see Cecil & McPhee (1959) and Danehy (1966)].

Insulin is a small protein hormone consisting of two polypeptide chains linked by two interchain disulfide bonds. A third intrachain disulfide bond is present in the A chain. The chemical reactivity of the cystine disulfide bridges have been reviewed (Blundell et al., 1972) and are implicated in insulin action (Clark & Harrison, 1982). The alkali cleavage of insulin and other proteins has been studied (Fønss-Bech & Nielsen, 1961; Cavallini et al., 1970a,b); however, the mechanism of this disulfide bond cleavage has not been fully described. In the following report, difference and first derivative spectroscopy and analysis of reaction stoichiometry of alkali-treated insulin are described in an attempt to clarify the mechanism of alkali disulfide bond cleavage in insulin.

Materials and Methods

Materials. Single peak porcine zinc insulin (0.3% w/w zinc; 25.7 units/mg) was obtained from Commonwealth Serum Laboratories, Melbourne, Victoria, Australia. Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)], cystine, and cysteine were from Sigma Chemical Co., St. Louis, MO. Sephadex gels and dextran blue were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. The A and B chains of insulin were prepared according to the method of Cecil & Loenig (1960). *N,N*-Dimethyl-*p*-phenylenediamine was synthesized from methyl orange according to the method of Vogel (1972) and was 3 times recrystallized from ether. All other materials were of reagent grade and obtained commercially.

Equipment. The "Alga" micrometer syringe was from Wellcome Co., Beckenham, England, and Seralute T4 columns were from Miles Laboratories Australia Pty. Ltd., Springvale, Victoria, 3171. The pH of solutions was estimated by using a wide-range (0–14) pH electrode (Radiometer type GK 2402B, Copenhagen, Denmark). Time or wavelength vs. absorbance spectra were recorded on a Varian Superscan 3 (735) ultraviolet–visible spectrophotometer at a scan rate of 100 nm/min, at a slit width of 1 nm, and with the aid of programmed base line.

Preparation of Insulin Solutions. Porcine zinc insulin was dissolved in 0.1 N HCl and was then passed through a sterile 0.2-μM Millipore filter. Aliquots (0.5 mL) of the filtered solution were stored frozen at −20 °C. The precise concentration of freshly thawed solutions of insulin was determined from the absorbance at 277.5 nm by using the extinction coefficient of 5.53×10^3 M^{-1} cm^{-1} as determined by Harrison & Garratt (1969).

Alkaline insulin solutions were prepared by the addition of KOH to a suitable amount of the insulin as prepared above. The pH of this solution was checked and adjusted as required with KOH, or HCl. Where solutions were kept "under N_2", they were 3 times degassed with a water pump and repurged with N_2. The temperature of the reaction solutions was 22 ± 3 °C.

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Hydroxide Ion Consumption. The amount of hydroxide ions consumed with time during the alkaline treatment of insulin was determined by titrating gravimetrically determined 1-mL aliquots of the reaction solution to pH 7 with standardized HCl. This was done by using a two-step procedure. One milliliter of 0.09 M HCl was first added, and the exact amount was determined gravimetrically. The titration to pH 7 was then completed by using an Alga micrometer syringe [fitted with a 12 cm length of fine bore (0.5 mm) delivery tubing] outfit which was gravimetrically standardized to deliver (0–300) ± 0.5 μL of 0.09 M HCl. The tube in which the titration was performed was of such a dimension that a magnetic stir bar, the delivery tube and pH electrode could all be fitted into the tube. They displaced enough of the neutralizing solution to cover the electrode adequately during stirring.

Sulfhydryl Group Analysis. The number of sulfhydryl groups was determined by a modified method of Habeeb (1972). At a selected time, 0.1 mL of the test solution was added to 2.5 mL of 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate, and 2.5 mM Ellman's reagent (DTNB) at pH 8.0. After 20 min at room temperature, the absorbance at 412 nm was measured against a freshly prepared reagent blank. The extinction coefficient of reduced DTNB at pH 8 was taken to be 13 600 M^{-1} cm^{-1} (Ellman, 1959; Habeeb, 1972; Kuramitsu & Hamaguchi, 1979). We also obtained this value from cysteine standard curves.

Cyanolyzable Sulfur and Sulfinic Acid Analyses. A method similar to the cold cyanolysis procedure of Sörbo (1957a,b) and Cavallini et al. (1960) as modified by Gawron & Odstrchel (1967) was used. A 0.1-mL aliquot of sample was mixed with 1 mL of 0.2 M borate, pH 9, and 0.5 mL of sodium cyanide, pH 9. After the sample stood at room temperature for 10 min, the thiocyanate formed was determined by using 0.4 mL of ferric reagent [100 g of $Fe(NO_3)_3 \cdot 9H_2O$, 200 mL of HNO_3 made to 1 L with H_2O). Protein precipitates were removed by centrifugation. The extinction coefficient of the ferrithiocyanate complex was determined from a KSCN standard curve to be 3860 M^{-1} cm^{-1} at 460 nm. Sulfinic acids also give a red color with ferric ions and could be distinguished from cyanolyzable sulfur by adding the ferric nitrate reagent to the sample before the cyanide (Sörbo, 1957b).

Sulfide Analysis. The colorimetric determination of methylene blue which is formed by the fusion of *N,N*-dimethyl-*p*-phenylenediamine (DMPDA) in the presence of sulfide and $FeCl_3$ is the basis of the method of Siegel (1965). A modified method was used for the quantitative determination of sulfide formed during the alkali lysis of insulin and/or by subsequent acid treatment. A 50-μL aliquot of sample (2 mM insulin in 0.1 M KOH and 0.05 M KCl at pH 13) was pipetted into the bottom of a 5 × 0.5 cm polyethylene test tube. The tube was then held horizontal, and the 50 μL of 0.3 M HCl was pipetted as a single droplet about one-third of the way out of the tube. Care was taken to avoid mixing the acid droplet and the sample. The tube, still held horizontal, was sealed with 0.4 mL of water placed in the upper one-third. The acid and sample droplets were mixed by tipping the tube upright. The trapped air within the tube isolates the water from the acid sample mix. After a preselected time of acid treatment, 50 μL of 0.03 M $FeCl_3$ in 1.2 M HCl and 50 μL of 0.02 M DMPDA in 7.2 M HCl were pipetted into a cap which was then fitted tightly over the top of the tube. Surface tension within the cap prevented premature mixing of the

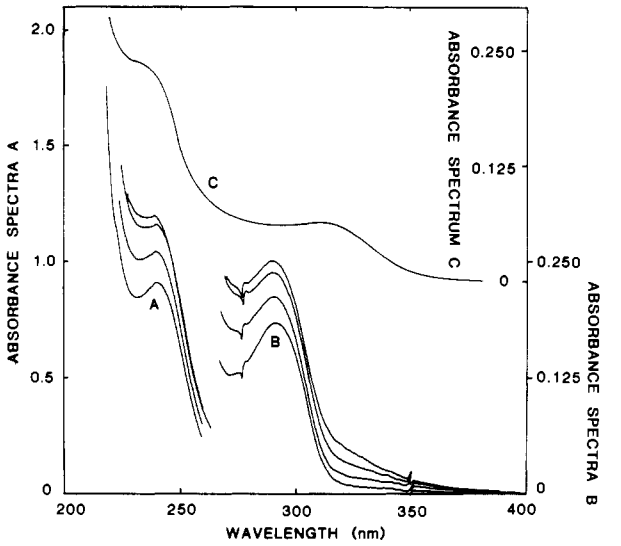

FIGURE 1: Time-dependent changes in the ultraviolet–visible absorption spectra of alkali-treated insulin. Spectra A and B were recorded for a 20 μM solution of insulin which was alkali treated for 0.5, 30, 75, and 150 min (lowest to highest intensity absorbance spectra, respectively) at pH 13 (0.1 M KOH, 0.05 M KCl), 22–26 °C, and under N_2. The spectra were recorded against a reference solution of 0.1 M KOH and 0.05 M KCl, and the times were recorded from the start of the 100 nm/min spectral scans. Spectrum C was the difference between the absorbance of the insulin solutions after 180 and 0.5 (reference) min of alkali treatment.

DMPDA/$FeCl_3$ reagent with the contents of the tube. The tube was next vortexed so that the acid-treated sample was diluted with the water and then inverted and vortexed so that the diluted sample was mixed with the DMPDA/$FeCl_3$ reagent. Reproducible results were achieved provided the DMPDA and $FeCl_3$ were added to each other just prior to use and that the DMPDA/$FeCl_3$ reagent was not mixed with the contents of the tube prior to the water dilution of the acid-treated sample. After 30 min in the dark, the tubes were opened and diluted with 0.5 mL of water. The tubes were centrifuged to remove insoluble protein, and then the absorbance at 668 nm was determined. The extinction coefficient at 668 nm was estimated from sodium sulfide standard curves to be 25.3 × 10^3 M^{-1} cm^{-1}.

Desalting. Seralute T4 columns were packed with 1-mL gel cakes of Sephadex G-25 superfine and used according to the microcentrifuge desalting technique of Helmerhorst & Stokes (1980).

Results

Time-dependent changes were observed in the absorption spectrum from 200 to 400 nm in solutions of insulin which were alkali treated above pH 12. Successive wavelength scans were recorded for up to 3 h for a 20 μM solution of insulin at pH 13 and are shown in Figure 1. The difference spectrum (Figure 1C) between the 0.5-min and 3-h scans revealed that two new peaks were generated with absorption maxima at 240 and 315 nm and with extinction coefficients of 16 × 10^3 and 2.9 × 10^3 M^{-1} cm^{-1}, respectively. Both chromophores chromatographed with the protein peak on Sephadex G-25 superfine and were not diminished when treated in 0.1 M KCN. No time-dependent changes were observed in a 20 μM solution of insulin which was alkali treated at pH 11.4 for 2 h.

Another less intense chromophore of different identity was observed when a more concentrated solution of insulin was alkali treated. The rate of absorbance change with change in wavelength was estimated for a 2 mM solution of insulin at pH 13 and illustrated that a new chromophore was gen-

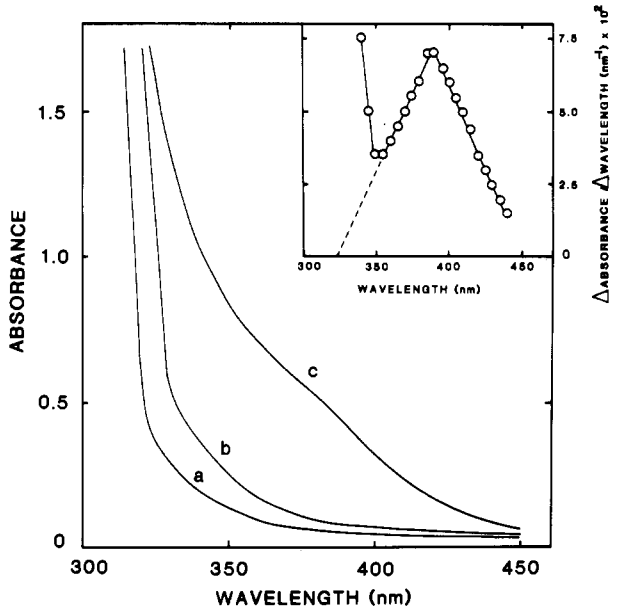

FIGURE 2: Appearance of a new absorbing peak in alkali-treated insulin which is removable by cyanide. Spectra were recorded for a 2 mM solution of insulin which was alkali treated for 0.5 min (a) and 4 h (c) at pH 13 (0.1 M KOH, 0.05 M KCl), 22 °C, and under N_2. The first derivative of the difference between curves a and c is plotted against the wavelength and is illustrated in the inset. Solid KCN (5 mg) was added per mL of insulin (c) and to the reference solution (0.1 M KOH, 0.05 M KCl), and the spectrum was recorded after 5 min (b). No further change in the spectrum (b) was observed after 30 min. Times were recorded from the start of the 100 nm/min spectral scans.

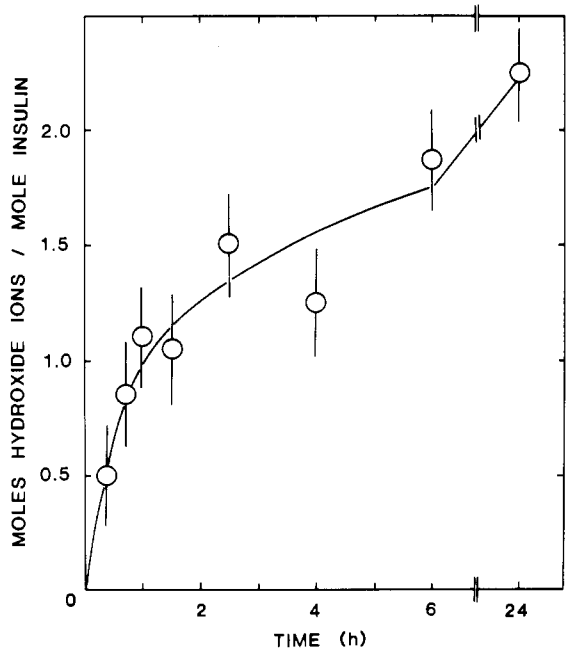

FIGURE 3: Stoichiometry of hydroxide ion consumption during the alkaline treatment of insulin. Insulin (2 mM) was alkali treated at pH 13 (0.1 M KOH, 0.05 M KCl), 23 °C, and under N_2. The amount of hydroxide consumed with time was determined as described under Materials and Methods. Each point represents the mean ± the standard error of four estimates which were obtained from two experiments. In addition, each point was corrected for a time-dependent drift of the blank titrations.

erated (Figure 2, inset) and was apparent as a broad shoulder between 350 and 400 nm (Figure 2c) in accordance with the initial observations of Cavallini et al. (1970a). Extrapolation to zero change in absorbance with change in wavelength

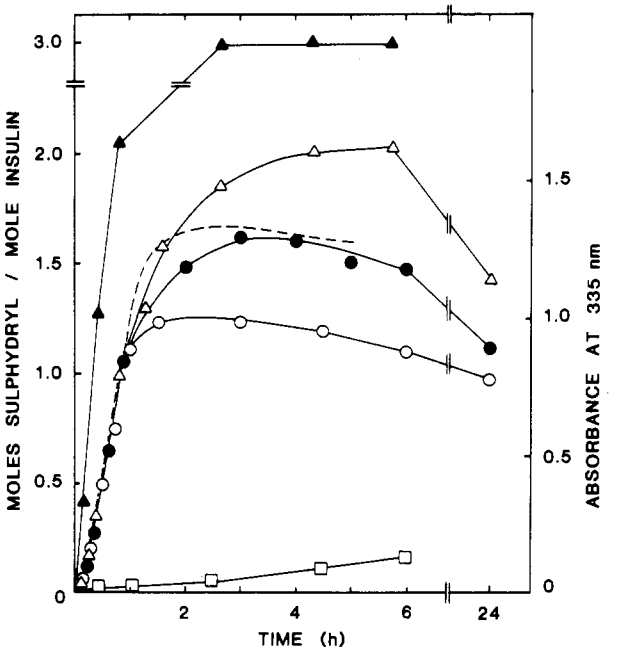

FIGURE 4: Generation of sulfhydryl groups in alkali-treated insulin. The number of sulfhydryl groups which were generated with time was determined as described under Materials and Methods for the following: 2 mM alkali-treated insulin at pH 13 (0.1 M KOH, 0.05 M KCl), 22 °C, and under N_2 [solution A (△)]; solution A made 7.5 M in urea (▲); 0.5 mM alkali-treated insulin at pH 12 (0.01 M KOH, 0.05 M KCl), 22 °C, and under N_2 (□). The number of sulfhydryl groups which were cyanolyzable at room temperature was determined as described under Materials and Methods for solution A (●) or solution A kept in air (○). The absorbance of solution A at 335 nm was recorded with time (---). All the estimates are expressed as the means of no less than three determinations, and the size of all the symbols are indicative of the errors in those determinations.

(Figure 2, inset) defined the peak absorbance of the new chromophore to be 323 nm. This chromophore was distinguished from the 315-nm chromophore which was reported above by (a) its lower extinction coefficient (670 M^{-1} cm^{-1}) and (b) the abolished absorbance of this chromophore by 0.1 M KCN (Figure 2b,c); there was a large residual absorbance at 315 nm after KCN treatment (Figure 2a,b). This low intensity chromophore was not detected when the alkali-treated insulin was acidified (pH 1); however, about 70% of the initial absorbance above 330 nm was regenerated when the acidified solution was again made alkaline (pH 13) (the precise value was difficult to estimate because of slowly and continuously appearing turbidity).

The observed spectral changes were associated with the consumption of hydroxide ions and generation of sulfhydryl groups. Within 6 h, up to 2 mol of hydroxide ion was consumed per mol of a 2 mM solution of insulin at pH 13, and no further consumption was detected after 24 h (Figure 3). There was a concomitant generation of 2 mol of sulfhydryl/mol of insulin within 6 h, followed by a slow decline over 24 h (Figure 4). A further 1 mol of sulfhydryl/mol of insulin was generated when the insulin was alkali treated in the presence of 7.5 M urea. The generation of sulfhydryl was dependent on the concentration of insulin and hydroxide ions. At pH 12 the generation of sulfhydryl in a 0.5 mM solution was 1/40 the rate of 2 mM insulin at pH 13.

When alkali-treated insulin was reacted with alkaline cyanide at room temperature and then was mixed with acidic ferric nitrate, a chromophore was generated (absorbance maximum at 460 nm) which was characterized of a ferrithiocyanate complex (Sörbo, 1957a,b). Similarly, sulfinic acid

Table I: Protein-Bound and Acid-Stable Cyanolyzable Sulfur Residue in Alkali-Treated Insulin Which Could Also Be Generated by Treating Insulin with Exogenous Thiol under Mild Conditions[a]

test	treatment	mol of cyanolyzable sulfur/mol of insulin
A	2 mM insulin under N_2 for 4 h at pH 13 (0.1 M KOH, 0.05 M KCl) and 22 °C	1.540 ± 0.031
B	solution A desalted	0.996 ± 0.021
C	solution A acidified at pH 1 for 1 h at 22 °C	1.002 ± 0.010
D	solution C desalted	1.006 ± 0.062
E	2 mM insulin for 4 h in 25 mM sodium borate, pH 9.0, 24 °C, and containing 50 mM cysteine	0.847 ± 0.104
F	2 mM insulin for 4.5 h in 25 mM sodium borate, pH 8.9, 24 °C, and containing 50 mM Na_2S	0.764 ± 0.091

[a] Solutions were desalted as described under Materials and Methods. All estimates are expressed as the mean ± the standard deviation of triplicate determinations.

and ferric ions form a red-colored chromophore with an absorbance maximum at 460 nm. This, however, did not account for the absorbance which was observed at 460 nm as less than 3% of the absorbance was developed if the ferric nitrate reagent was added to the alkali-treated insulin prior to the KCN (see Materials and Methods for sulfinic acid analysis). Thus, when 2 mM insulin was alkali treated at pH 13 in air or under N_2, approximately 1.3 and 1.5 mol of sulfur, respectively, were cyanolyzable at room temperature (Figure 4). Only 1 mol of cyanolyzable sulfur was detected after 24 h of alkali treatment in either sample. Neither cysteine, cystine, sulfide, nor inorganic sulfur was cyanolyzable under the same conditions.

The initial rates of sulfhydryl and cyanolyzable sulfur group generation were similar and diverged only when more than 1 mol of either group was generated per mol of insulin (Figure 4). As the second mole of sulfhydryl was generated, proportionately less of the cyanolyzable sulfur was detected, and this was especially marked when the samples were exposed to air. In addition, an initial lag period was observed during the generation of sulfhydryl and cyanolyzable sulfur groups, and this has also been observed by others (Cavallini et al., 1970a) when the absorbance of the alkali-treated insulin was monitored at 335 nm (also see Figure 4).

One mole of cyanolyzable sulfur was detected per mol of a 2 mM solution of alkali-treated insulin which had been desalted (Table I, test A plus B), and it was associated with the protein fraction. Furthermore, 1 mol of cyanolyzable sulfur was detected per mol of alkali-treated insulin which had been acidified for 1 h at pH 1, and its similarly eluted with the protein fraction when desalted on Sephadex G-25 superfine (Table I, test C and D). Moreover, acid-stable and protein-associated cyanolyzable sulfur was generated in solutions of insulin which were treated under mild conditions (pH 9) with either cysteine or sulfide (Table I, test E plus F). In these experiments no cyanolyzable sulfur was detected without added cysteine or sulfide.

Immediately as the alkali-treated insulin (Table I, test C) was acidified, a strong smell of hydrogen sulfide was observed. The amount of sulfide evolved was dependent on the time of alkaline treatment of the insulin, and 0.2 mol of sulfide was detected per mol of insulin after 4 h (Figure 5). The evolution of 0.2 mol of sulfide correlated with a decrease of 0.5 mol of cyanolyzable sulfur/mol of insulin. A particularly marked lag was noticeable during the production of sulfide.

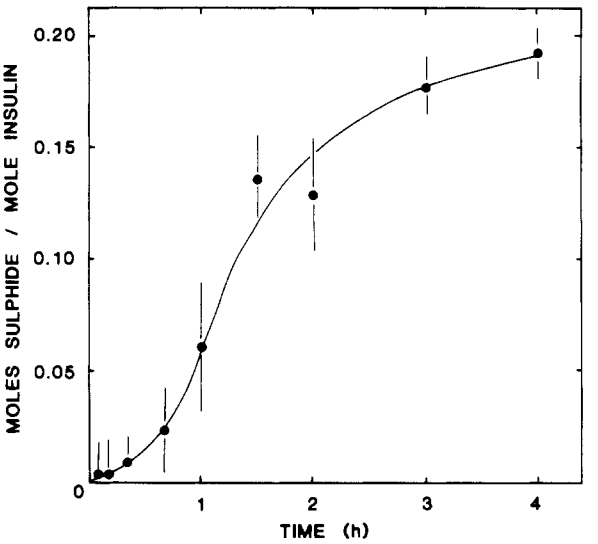

FIGURE 5: Evolution of sulfide when alkali-treated insulin was acidified. The amount of sulfide which was liberated when 2 mM solutions of insulin were first alkali treated at pH 13 (0.1 M KOH, 0.05 M KCl), 22 °C, and under N_2 for varying periods of time and then acidified for 1 h was determined as described under Materials and Methods. No more sulfide was detected when any of the solutions were acidified for longer than 1 h. Each point was expressed as the mean ± the standard deviation of triplicate determinations.

Gel filtration chromatography of alkali-treated insulin on a column of Sephadex G-50 illustrated that fragmentation of insulin had occurred, yielding two components which co-chromatographed with reduced insulin A and B chains (about half of the absorbing material) and a third component of even lower apparent molecular weight (Figure 6A). A smaller peak of apparent molecular weight greater than native insulin was also observed. The species with the lowest apparent molecular weight, corresponding to about a third of the total absorbance at 277.5 nm, was transformed after 1 h of acid treatment to higher apparent molecular weight, and no net change in the A and B chainlike components was observed. However, after 30 h of acid treatment, some of the A chainlike component was also transformed to higher apparent molecular weight.

Discussion

Two mechanisms are commonly invoked to explain disulfide bond fission of proteins or low molecular weight model compounds in alkali.

$$2R\text{—}S\text{—}S\text{—}R + 4OH^- \overset{A}{\rightleftharpoons} 2RS^- + 2RSOH + 2OH^- \overset{B}{\rightleftharpoons} 3RS^- + RSO_2^- + 2H_2O \quad (1)$$

$$R\text{—}S\text{—}S\text{—}CH_2\text{-}C\text{-}R_2H + OH^- \rightleftharpoons R\text{—}S\text{—}S^- + CH_2{=}CR_2 + H_2O \quad (2)$$

The alkaline hydrolysis mechanism (1) involves the direct attack of hydroxide ions on the sulfur atom (Schöberl, 1933; Speakman, 1933) with subsequent cleavage of the disulfide to a thiol and sulfenic acid (1A). The sulfenic acid is unstable (Challenger & Rawlings, 1937) and reacts further to form thiol and sulfinic acid (1B) or sulfonic acid (Donovan & White, 1971). The data for the alkaline lysis of aliphatic disulfides (Danehy & Hunter, 1967; Wronski, 1963) and of ovomucoid (Donovan, 1967a,b) are reported to be consistent with this reaction stoichiometry.

According to reaction mechanism 2, hydroxide ions initiate the cleavage of C–S bonds by abstracting hydrogen from the carbon β to the sulfur atom (Tarbell & Harnish, 1951; Swan, 1957; Cecil & McPhee, 1959). This yields persulfide and

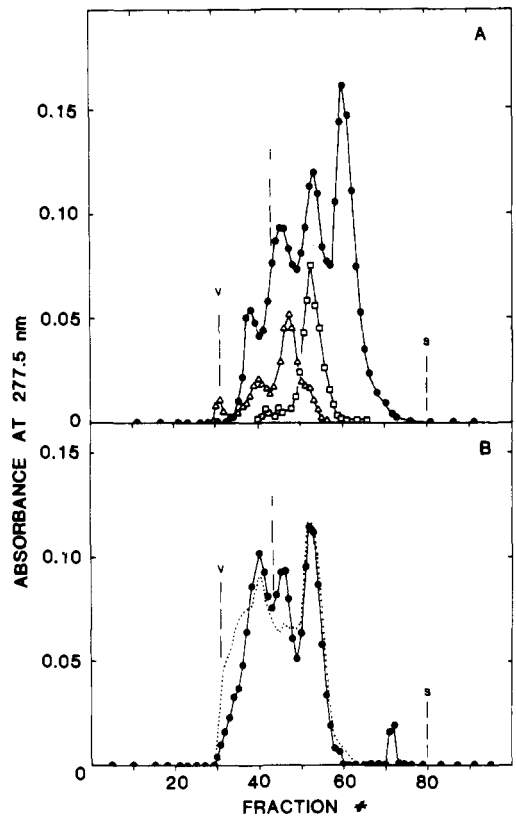

FIGURE 6: Fragmentation of alkali-treated insulin and its aggregation in acid. A column (1.5 × 54.5 cm) of Sephadex G-50 superfine was equilibrated and eluted with 0.2 M Tris in 50% methanol at pH 8.0 containing 7.5 M urea. Samples (1 mL) were applied in the same buffer, and the absorbance of the eluate at 277.5 nm was monitored for each 50-drop (1.2 mL) fraction. The void (*v*), salt (s), and insulin (*i*) elution volumes were determined with dextran blue, potassium chromate, and 0.5 mM insulin (untreated), respectively. Insulin A (△) and B (□) chains were prepared as described under Materials and Methods. A 0.25-mL aliquot of 2 mM insulin, which was alkali treated for 4 h at pH 13 (0.1 M KOH, 0.05 M KCl), 24 °C, and under N_2, was diluted with 0.75 mL of 4/3 strength elution buffer and then chromatographed (A) (●). Another 1-mL aliquot of the alkali-treated insulin was mixed with an equal volume of 0.3 M HCl, and after 1 h (B) (●) or 30 h (B) (···), 0.5 mL of the acidified sample (0.1 M HCl, 0.075 M KCl) was diluted with 0.5 mL of double-strength elution buffer and then chromatographed.

dehydroalanine (aminoacrylate) residues. Evidence supporting this mechanism has been presented for several aliphatic disulfides (Rao & Gorin, 1959; Villarejo & Westley, 1963; Federici et al., 1977) and proteins (Bohak, 1964; Schneider & Westley, 1969; Cavallini et al., 1970a,b).

When insulin was treated with alkali, time-dependent increases in absorbance were observed at 240, 315, and 323 nm (Figures 1 and 2) with apparent extinction coefficients of 16.0 × 10^3, 2.9 × 10^3, and 670 M^{-1} cm^{-1}, respectively. In contrast to the conclusions drawn by Donovan (1967a) from his study on alkali-treated ovomucoid, the generation of sulfhydryl in alkali-treated insulin did not alone account for the increased absorbance at 240 nm. The 2 mol of sulfhydryl which was generated per mol of alkali-treated insulin (Figure 4) would have accounted for only 62% of the observed absorbance change (the extinction coefficient of sulfhydryl at 240 nm was taken to be 5 × 10^3 M^{-1} cm^{-1}; Donovan, 1973). Furthermore, as neither sulfenic, sulfinic, nor sulfonic acid groups absorb appreciably near 240 nm (Donovan & White, 1971), the increase in absorbance at 240 nm for alkali-treated insulin was inconsistent with the stoichiometry of reaction mechanism 1.

The absorption spectrum of dehydroalanine closely follows that of sulfhydryl ion (Carter & Greenstein, 1946), and the extinction coefficient of glycyldehydroalanine is 4 × 10^3 M^{-1} cm^{-1} at 240 nm. According to reaction mechanism 2, the 2 mol of sulfhydryl produced per mol of alkali-treated insulin would initially be persulfide residues (RSSH), and 2 mol of dehydroalanine residues would also arise as the other reaction product. Dehydroalanine residues would therefore contribute 50% of the increased absorbance at 240 nm and persulfide residues the remainder. Certainly, alkaline inorganic persulfide, sulfide, and elemental sulfur show strong absorbance at 240 nm (Federici et al., 1977). In addition, some alkaline degradation products of persulfide may have contributed to the absorbance change at 240 nm. Nevertheless, the absorbance change of alkali-treated insulin at 240 nm would be consistent with the stoichiometry of reaction mechanism 2.

The product of alkali-treated insulin which was chromogenic at 315 nm was not identified. However, it is interesting to note that the spectra A–C of Figure 1 are very similar to the spectra of alkali-treated ovomucoid which were illustrated by Donovan (1967a), who also was unable to identify the chromophore absorbing above 270 nm.

Persulfide ions have been tentatively reported as a product of alkaline-treated cystine (Rao & Gorin, 1959), lipoic acid (Villarejo & Westley, 1963), and oxidized glutathione (Schneider & Westley, 1969; Federici et al., 1977) and have been identified with a weak chromophore at 335 nm. An extinction coefficient of 100 M^{-1} cm^{-1} was calculated for this chromophore from Schneider & Westley's (1969) data for the alkaline hydrolysis of oxidized glutathione. Schneider & Westley (1969) reported a spectral change for insulin treated with 0.5 M NaOH, and the new chromophore showed an apparent absorption maximum at 370 nm. The chromophore was abolished by cyanide and was not detected when a mixture of fully reduced insulin A and B chains was alkali treated. These results implied that the generation of a cyanolyzable chromogen was dependent on disulfide bond lysis. Cavallini et al. (1970a,b) verified the generation of a cyanolyzable chromophore in alkali-treated insulin and also reported broad absorption bands (300–450 nm) for alkali-treated ribonuclease, bovine serum albumin, and other proteins. A first derivative difference spectrum of the broad shoulder on the tyrosyl absorption (Figure 2, inset) was extrapolated to the absorbance peak at 323 nm. In agreement with the results of Schneider & Westley (1969), we observed that cyanide abolished this absorbance at 323 nm (Figure 2), and under the conditions of our test, 1.5 cyanolyzable residues were detected (Table I and Figure 4). Therefore, the cyanolyzable chromogen had an extinction coefficient of 420 M^{-1} cm^{-1} and is assigned to the persulfide which would be generated according to reaction mechanism 2.

It is unlikely that the ultraviolet absorbance changes of alkali-treated insulin were due to the oxidation of tyrosyl residues as proposed by Inada (1961) for the following reasons: cyanide abolished the absorbance at 323 nm, the time-dependent changes occurred at a faster rate under N_2, tyrosyl oxidation generates more intense chromophores, some of which are centered at longer wavelengths than we observed, and furthermore, the tyrosyl residues of insulin are relatively resistant to oxidation (Haas et al., 1951). These observations confirm those of Frank et al. (1972) and Garratt & Walson (1967).

Gel filtration of alkali-treated insulin (Figure 6) illustrated that the hormone was fragmented into at least four components, two of which cochromatographed with reduced A and B chains of insulin. For this to occur, the two interchain disulfide bonds of insulin must have been cleaved. The in-

trachain disulfide bond of insulin was probably intact because a further sulfhydryl residue was cleaved when insulin was alkali-treated in 7.5 M urea (Figure 4). These observations were consistent with the reported reactivities of insulin's disulfide bonds [for review, see Blundell et al. (1972)]. Lysis of the two interchain disulfide bonds of insulin was associated with the consumption of two hydroxide ions (Figure 3) and the generation of two sulfhydryl residues (Figure 4). The analysis of the reaction stoichiometry of alkali-treated insulin was, therefore, consistent with reaction mechanism 2.

Schneider & Westley (1969) reported the generation of cyanolyzable sulfur residues from insulin in 0.5 M NaOH but not in 0.08 M NaOH. Cavallini et al. (1970a) reported cyanolyzable sulfur residues in solutions of insulin in 0.05 M NaOH. However, under their conditions of cyanolysis (CN in 0.01 M NaOH for 10 min), the direct nucleophilic attack of cyanide on the disulfide bonds of insulin may have contributed substantially to the rate of disulfide bond lysis (Schneider & Westley, 1969; Catsimpoolas & Wood, 1964). Moreover, cyanolyzable sulfur was measured as a ferrithiocyanate chromogen, and the appropriate control to check for other interfering Fe–chromogens was not reported by the aforementioned workers. Free sulfinic acid has been shown to form a Fe–chromogen (Sörbo, 1957b). Protein-bound sulfinic acid would be the product of alkali-treated insulin if cleavage occurred by the alkali hydrolysis mechanism (mechanism 1). This product may also have formed a Fe–chromogen if the chemical environment of the residue was conducive. Interfering Fe–chromogens contributed less than 4% to the absorbance which was observed in our analyses of cyanolyzable sulfur in alkali-treated insulin. In these experiments we detected approximately 1.5 mol of cyanolyzable sulfur/mol of insulin treated with 0.1 M KOH, under conditions where the direct attack of cyanide on the disulfide bonds was minimal (Table I and Figure 4; low zero time control).

The generation of cyanolyzable sulfur in alkali-treated proteins is indicative of a product with a polysulfide structure (R'-S_n-R, where $n > 1$, R may be H) (Cavallini et al., 1960). Persulfides (R–SS–H) have not been isolated, and only circumstantial evidence has been obtained for their existence. Nevertheless, persulfide residues have been proposed as functional intermediates in the active sites of several enzymes, including xanthine oxidase (Massey & Edmondson, 1970), aldehyde oxidase (Branzoli & Massey, 1974), rhodanese (thiosulphate:cyanide sulfurtransferase) (Weng et al., 1978), and cystathionase (Cavallini et al., 1960; Yamanishi & Tuboi, 1981). The cleavage of cystine by cystathionase generated a readily cyanolyzable species which was stable at 100 °C or in acid and was partially recovered after chromatography on Dowex 50 (Cavallini et al., 1960). The species was tentatively identified as a persulfide and was called thiocysteine. However, the stability of thiocysteine was at variance with the predicted reactivity of persulfide (reactions 3 and 4).[1] Cavallini et al.

$$R—S—S^- \rightleftharpoons R—S^- + S \quad (3)$$

$$2R—S—S^- \rightleftharpoons R—S—S—R + S^{2-} + S \quad (4)$$

(1960) concluded that either the persulfide survived degradation or it was converted to an acid-stable and readily cyanolyzable compound of unknown nature. A residue with similar properties was observed when alkali-treated insulin was

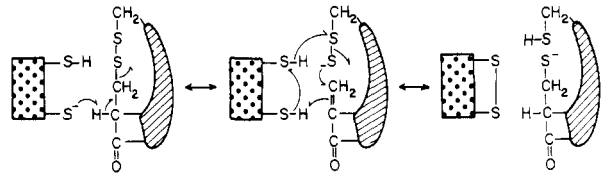

FIGURE 7: Proposed mechanism for intermediate persulfide formation in thiol–disulfide interchange.

acidified. One mole of protein-bound cyanolyzable sulfur survived the acidification (Table I). This acid-stable persulfide-like residue may be similar to the trisulfide described by Abdolrasulnia & Wood (1980). The remaining 0.5 mol of cyanolyzable sulfur/mol of insulin was unstable to acid treatment. In addition, a distinct smell of hydrogen sulfide was detected from the acidified solution of alkali-treated insulin. According to reaction 4, the generation of 0.25 mol of sulfide and a 33% incorporation of species absorbing at 277.5 nm into higher molecular weight adducts would be predicted. Indeed, analysis of sulfide (Figure 5) demonstrated that up to 0.2 mol of the gas had been evolved and the species with lowest apparent molecular weight, corresponding to one-third of the total absorbance at 277.5 nm, was transformed after acid treatment to higher apparent molecular weight (Figure 6). Furthermore, the absorbance at 335 nm decreased one-third when alkali-treated insulin was acidified, and this observation was consistent with the chromogenicity and partial acid lability of the cyanolyzable sulfur. These results account for the degradation of the unstable cyanolyzable sulfur in acid according to reaction 4 and indirectly support the generation of persulfide-like residues in alkali-treated insulin. However, only 80% and 65% of the sulfhydryls were detected as cyanolyzable sulfur in alkali-treated insulin under N_2 or in air, respectively. This implied that some of the unstable cyanolyzable sulfur was also decomposed with time in alkali to noncyanolyzable sulfhydryl possibly via reaction 3 as only this mechanism would preserve total sulfhydryl.

The time lag of sulfide generation (Figure 5) was particularly marked. Less than 25% of the total sulfide was detected when the first mole of cyanolyzable sulfur had been generated. Furthermore, the values of total sulfhydryl and cyanolyzable sulfur did not diverge until more than 1 mol of cyanolyzable sulfur had been generated per mol of insulin. The rapid generation of an acid-stable cyanolyzable sulfur residue and the slow generation of an unstable cyanolyzable sulfur residue would explain these observations and would also be consistent with the observed lags in the generation of sulfhydryl and cyanolyzable sulfur and absorbance at 335 nm (Figure 4). The lysis of the fast-reacting interchain disulfide bond of insulin might be necessary to expose the second interchain disulfide bond, and this would explain the observed lag in latent hydrogen sulfide. The least protected disulfide group of insulin is the A7–B7 interchain bridge which has its sulfur atoms accessible to the solvent in all states of aggregation[2] and is easily available to attack by nucleophilic chemical reagents [for review, see Blundell et al. (1972)]. The A20–B19 interchain disulfide is much less exposed and may be a slower reacting residue.

We propose that thiol–disulfide interchange in insulin may proceed by the generation of a persulfide intermediate as illustrated in Figure 7. Like hydroxide ions, thiolate ions (RS^-) interact with accessible disulfide bonds of proteins and

[1] Similarly, cyanolyzable sulfur has been reported to be generated by the action of excess sulfur on alkaline thiol (Hylin & Wood, 1959), or excess sulfide on alkaline disulfide (Cavallini et al., 1970b).

[2] Depending on conditions of pH, ionic strength, solvent, etc., insulin self-associates in solution to form complex mixtures of monomers, dimers, tetramers, and hexamers.

generate persulfide-like residues. Cavallini et al. (1970b) monitored the absorbance changes at 335 nm and concluded that persulfide residues were generated by sulfide in urea-denatured bovine serum albumin at pH as low as 8–9. The disulfide bonds of urea-denatured insulin were reported to be twice as reactive with sulfide as those of urea-denatured bovine serum albumin or cystamine. Similarly, we found that the disulfide bonds of insulin were sensitive to thiolate ions because cyanolyzable sulfur was generated when native insulin was treated with cysteine or sulfide at pH 9 (Table I). In addition, it has been shown that the disulfide bonds of insulin are reduced with the concomitant oxidation of the active site cysteine residues of thioredoxin (Holmgren, 1979) or *N*-acetyltransferase (Namboodiri et al., 1981). Moreover, it has been demonstrated that insulin forms a mixed disulfide complex with its receptor as a result of thiol–disulfide interchange (Clark & Harrison, 1982).

We have recently demonstrated the production of dehydroalanine in insulin by NMR as the time-dependent appearance of resonances clustered around 5.5 ppm (A. J. Jones, E. Helmerhorst, and G. B. Stokes, unpublished results). These resonances were assigned by using alkali-treated oxidized glutathione as a model compound (Asquith & Carthew, 1972). We conclude from anayses of time-dependent spectral changes, the consumption of hydroxide ions, the generation of sulfhydryl groups, and the persulfide-like nature of the products of alkali-treated insulin that the β-elimination mechanism accounts for alkaline disulfide bond lysis in this protein.

To our knowledge, this is the first report of an acid- and alkali-stable, protein-associated persulfide residue. The chemical environment around this residue in insulin may have a stabilizing effect. Alkali or sulfhydryl-treated insulin is then an excellent model for future studies of persulfide residues in proteins. Work is continuing to determine the site and nature of this acid-stable persulfide-like moiety in insulin.

Registry No. Insulin, 9004-10-8.

References

Abdolrasulnia, R., & Wood, J. L. (1980) *Bioorg. Chem. 9*, 253–260.

Asquith, R. S., & Carthew, P. (1972) *Biochim. Biophys. Acta 285*, 346–351.

Blundell, T., Dodson, G., Hodgkin, D., & Mercola, D. (1972) *Adv. Protein Chem. 26*, 279–402.

Bohak, Z. (1964) *J. Biol. Chem. 239*, 2878–2887.

Branzoli, U., & Massey, V. (1974) *J. Biol. Chem. 249*, 4346–4349.

Carter, C. E., & Greenstein, J. P. (1946) *J. Biol. Chem. 165*, 725–726.

Catsimpoolas, N., & Wood, J. L. (1964) *J. Biol. Chem. 239*, 4132–4137.

Cavallini, D., De Marco, C., Mondovi, B., & Mori, B. G. (1960) *Enzymologia 22*, 161–173.

Cavallini, D., Federici, G., Barboni, E., & Marcucci, M. (1970a) *FEBS Lett. 10*, 125–128.

Cavallini, D., Federici, G., & Barboni, E. (1970b) *Eur. J. Biochem. 14*, 169–174.

Cecil, R., & McPhee, J. R. (1959) *Adv. Protein Chem. 14*, 255–389.

Cecil, R., & Loening, U. E. (1960) *Biochem. J. 76*, 146–154.

Challenger, F., & Rawlings, A. A. (1937) *J. Chem. Soc.*, 868–875.

Clark, S., & Harrison, L. C. (1982) *J. Biol. Chem.* (in press).

Danehy, J. P. (1966) in *The Chemistry of Organic Sulphur Compounds* (Kharasch, N., & Meyers, C. Y., Eds.) Vol. 2, Chapter 13, Pergamon Press, Oxford.

Danehy, J. P., & Hunter, W. E. (1967) *J. Org. Chem. 32*, 2047–2053.

Donovan, J. W. (1967a) *Biochem. Biophys. Res. Commun. 29*, 734–740.

Donovan, J. W. (1967b) *Biochemistry 6*, 3918–3923.

Donovan, J. W. (1973) *Methods Enzymol. 27*, 497–548.

Donovan, J. W., & White, J. M. (1971) *Biochemistry 10*, 32–38.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Federici, G., Duprê, S., Matarese, R. M., Solinas, S. P., & Cavallini, D. (1977) *Int. J. Pept. Protein Res. 10*, 185–189.

Fønss-Bech, P., & Nielsen, M. D. (1961) *Rep. Steno Mem. Hosp. Nord. Insulinlab. 10*, 127–137.

Frank, B. H., Veros, A. J., & Pekar, A. H. (1972) *Biochemistry 11*, 4926–4931.

Garratt, C. J., & Walson, P. (1967) *Biochem. J. 105*, 51C–52C.

Gawron, O., & Odstrchel, G. (1967) *J. Am. Chem. Soc. 89*, 3263–3267.

Haas, W. J., Sizer, I. W., & Loofbourow, J. R. (1951) *Biochim. Biophys. Acta 6*, 589–600.

Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457–464.

Harrison, D. M., & Garratt, C. J. (1969) *Biochem. J. 113*, 733–734.

Helmerhorst, E., & Stokes, G. B. (1980) *Anal. Biochem. 104*, 130–135.

Holmgren, A. (1979) *J. Biol. Chem. 254*, 9113–9119.

Hylin, J. W., & Wood, J. L. (1959) *J. Biol. Chem. 234*, 2141–2144.

Inada, Y. (1961) *J. Biochem. (Tokyo) 49*, 217–225.

Kuramitsu, S., & Hamaguchi, K. (1979) *J. Biochem. (Tokyo) 85*, 443–456.

Massey, V., & Edmondson, D. (1970) *J. Biol. Chem. 245*, 6595–6598.

Namboodiri, M. A. A., Favilla, J. T., & Klein, D. C. (1981) *Science (Washington, D.C.) 213*, 571–573.

Rao, G. S., & Gorin, G. (1959) *J. Org. Chem. 24*, 749–753.

Schneider, J. F., & Westley, J. (1969) *J. Biol. Chem. 244*, 5735–5744.

Schöberl, A. (1933) *Justus Liebigs Ann. Chem. 507*, 111–127.

Siegel, L. M. (1965) *Anal. Biochem. 11*, 126–132.

Sörbo, B. (1957a) *Biochim. Biophys. Acta 23*, 412–416.

Sörbo, B. (1957b) *Biochim. Biophys. Acta 24*, 324–329.

Speakman, J. B. (1933) *Nature (London) 133*, 930.

Swan, J. M. (1957) *Nature (London) 179*, 965.

Tarbell, D. S., & Harnish, D. P. (1951) *Chem. Rev. 49*, 1–90.

Villarejo, M., & Westley, J. (1963) *J. Biol. Chem. 238*, 4016–4020.

Vogel, A. I. (1972) *A Textbook of Practical Organic Chemistry*, 3rd ed., pp 624–625, Lowe and Brydone, London.

Weng, L., Heinrikson, R. L., & Westley, J. (1978) *J. Biol. Chem. 253*, 8109–8119.

Wronski, M. (1963) *Monatsh. Chem. 94*, 197–203.

Yamanishi, T., & Tuboi, S. (1981) *J. Biochem. (Tokyo) 89*, 1913–1921.