filtered. This solid consisted of a mixture of the enol benzoate of 2-phenacyl-6-methylpyridine and the lithium salt of 2-phenacyl-6-methylpyridine. Soxhlet extraction with 60–70° petroleum ether separated the mixture into its components, the enol ester being soluble and the lithium salt being insoluble in petroleum ether. The original filtrate was extracted with several portions of ether, the combined ether phases dried over sodium sulfate, the ether removed at atmospheric pressure and the residue distilled in vacuum. In this manner, from several experiments, there were obtained 14.1–14.7 g. (44.8–46.7%) of the enol benzoate of 2-phenacyl-6-methylpyridine, m.p. 137.5–138.5°[4,5]; 10.0–10.1 g. (46.1–46.5%) of the lithium salt of 2-phenacyl-6-methylpyridine and 1.1–1.3 g. (5.2–6.2%) of 2-phenacyl-6-methylpyridine, b.p. 152–154° at 1.7 mm., m.p. 77–78°[3] (from 60–70° petroleum ether).

**Structure of the Enol Benzoate of 2-Phenacyl-6-methylpyridine.**—A sample (0.4 g.) of the enol benzoate was warmed for one minute with 25 ml. of 25% hydrochloric acid. On cooling the solution, a white solid precipitated and was filtered. The solid was shown to be benzoic acid (0.15 g., 97%), m.p. 120.6–121.4° alone and when mixed with an authentic sample. The filtrate was made basic with 5% sodium bicarbonate solution, extracted with ether and the ether removed to give 0.25 g. (93%) of 2-phenacyl-6-methylpyridine, m.p. 76.5–77.8°; picrate, m.p. 180–181° dec.[3]

**The Identity of the Lithium Salt of 2-Phenacyl-6-methylpyridine.**—When a sample of this material was placed in a flame, the color of the flame became bright red, indicative of the presence of lithium ions, after the sample carbonized. The identity of the salt was established further by dissolving it in hydrochloric acid and neutralizing the solution with 5% sodium carbonate solution. The mixture was cooled in an ice-bath and the solid which precipitated then was separated by filtration. The solid was air-dried and recrystallized from 60–70° petroleum ether. It melted at 76.5–78° and gave a picrate, m.p. 180–181° dec. A mixed melting point between this picrate and that prepared from an authentic sample of 2-phenacyl-6-methylpyridine showed no depression.

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CONTRIBUTION NO. 948
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF PITTSBURGH
PITTSBURGH 13, PENNA.

## The Reduction of the Disulfide Bonds of Insulin

BY H. LINDLEY

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Pierce[1] has recently reported the separation of the A and B chains of oxidized insulin by countercurrent procedures and shown that these two components almost certainly account for the whole of the insulin molecule. A similar conclusion has been reached in these laboratories by use of paper electrophoretic techniques on reduced insulin. Reduction offers theoretical advantages in that it is more specific and easily controlled than oxidation, but in the case of insulin suffers from the disadvantage that the products are insoluble in aqueous solutions except at extremes of *p*H. This difficulty has been overcome in the present work by the use of 8 *M* urea solutions. If insulin is reduced by 0.1 *M* lithium thioglycolate at *p*H 5 in the presence of 8 *M* urea and electrophoresis on paper is carried out with the same solution as supporting electrolyte, two components can be detected. By elution from the paper followed by hydrolysis and paper chromatography it was shown that the amino acid composition of the anodic component agreed qualitatively with that given by Sanger for the A chain whilst the cathodic component showed similar qualitative agreement with Sanger's B chain.

Evidence has been given by other workers that in the absence of urea approximately one-third of the cystine disulfide bonds of insulin are reduced at *p*H 5.[2,3] This was confirmed in the present work for specific reducing conditions used. This could be interpreted to imply either a limited reduction of all the disulfide bonds or alternatively a specific reduction of one sulfur bond of the three dissimilar bonds in the insulin molecule. There is considerable experimental evidence in the analogous case of wool to suggest that the latter alternative is more probable.[4]

Specific evidence for this view was obtained by reducing insulin at *p*H 5 with lithium thioglycolate and coupling the thiol groups so formed with iodoacetamide to give combined carbamyl-S-methylcysteine residues in the insulin molecule. Reduction of this product by lithium thioglycolate at *p*H 5 in the presence of 8 *M* urea and subsequent electrophoresis caused separation into two components which were isolated separately. By hydrolysis and paper chromatography it was shown that a strong spot corresponding in position to that expected for carboxy-S-methylcysteine occurred in the hydrolysate of the anodic fraction whereas the cathodic component gave only a very weak spot. This provides strong evidence that the major reaction which occurs during the reduction of insulin by thioglycolate solutions at *p*H 5 is fission of the intrachain disulfide bond of the A chain.[5]

The structure of insulin proposed by Lindley and Rollett[6] provides possible alternative explanations for the enhanced reactivity of this particular disulfide bond. In this proposed structure, the amino acid sequences of the A and B chains as determined by Sanger and collaborators[5,7,8] have been used and the polypeptide chains have been given essentially the configuration of an $\alpha$ helix.[9] In the particular arrangement of helices which has been proposed,[6] the intrachain disulfide bond of the A chain is more accessible to reagents than either of the interchain disulfide bonds. Alternatively, an unbonded NH group is present in the proposed structure in the vicinity of the intrachain disulfide bond and could create enhanced reactivity by stabilizing an intermediate stage in a single electron transfer mechanism of reduction. If this latter suggestion is correct then reduction at *p*H 5 may provide a simple chemical means of estimating intrachain disulfide bonds in which the two halves of the cystine residue are separated by compara-

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tively few other amino acid residues, since there is no *a priori* reason to suppose that interchain disulfide bonds necessitate unbonded main chain NH groups.[6,10] Evidence has already been obtained that this differentiation of the combined cystine into fractions of differing reactivity is not confined to wool but extends to other proteins.[2] Moreover, the fact that reduced wool reacts with formaldehyde to give some combined thiazolidine carboxylic acid[11] and the suggestion that oxidation of wool under some conditions leads to the formation of an internal sulfonamide,[12] both strongly suggest that some of the imino groups of the combined cystine are unusually reactive. The simplest explanation of this would be that these imino groups are not H-bonded.

If the chemical reactivity of the combined cystine of a protein can be simply related to structure in this way, this provides an important clue to the structure of proteins containing cystine. It could be of especial importance for lysozyme, ribonuclease and serum albumin and would imply the need for some revision of current ideas of wool and $\alpha$-keratin structure.

## Experimental

(a) **General Techniques.**—The technique of paper electrophoresis used was a simplified version of that described by Kunkel and Tiselius.[13] It was found advantageous to grease lightly the supporting glass plates with silicone lubricant and to bind them together with cellophane tape.[14] The paper was thoroughly wetted with the supporting buffer solution and after quickly removing surplus solution by light blotting, the protein solution was applied to an area which had been more completely dried by strong local blotting.[14] After electrophoresis the urea and lithium thioglycolate could be substantially removed from the paper by washing in three changes of denatured alcohol. As neither insulin nor its reduction products stained well with any of the reagents normally used for detecting protein components after paper electrophoresis, the Pauly test was used in this work.[15] The paper was sprayed with a freshly prepared 0.1% solution of diazotized sulfanilic acid in 5% sodium carbonate. The anodic component (A chain) gave a pink spot because of its tyrosine content whereas the color given by the cathodic component was more orange because of the simultaneous presence of both histidine and tyrosine. For work involving elution of products a thick commercial blotting paper was used. This was extracted prior to use by chromatographic washing with dilute ($N$) ammonia solution. Location of the components in these cases was carried out by using a second strip of Whatman No. 1 paper in contact with the main strip during the electrophoresis and using this after spraying as a guide.

(b) **Evidence of the Nature of the Reduction of Insulin at *p*H 5.**—3.5 mg. of crystalline insulin (Eli Lilly Lot No. 2842) was weighed into a 15-ml. centrifuge tube and dissolved in 0.2 ml. of 0.04 $N$ HCl. It was precipitated in amorphous form by neutralization with 0.2 ml. of 0.04 $N$ LiOH, and 0.1 ml. of 0.5 $M$ lithium thioglycolate solution was added and the mixture left for 4 hours. At the end of this period 10 ml. of acid acetone (1 ml. of $N$ HCl + 39 ml. of acetone) was added and the precipitated insulin centrifuged down. The thiol groups were then coupled by adding an excess of recrystallized iodoacetamide freshly dissolved in 0.5 ml. of 0.07 $M$ phosphate buffer of *p*H 7.15. After 1 hour the modified insulin was precipitated with 15 ml. of acid acetone and centrifuged off. The precipitate was dissolved in 0.1 ml. of 0.1 $M$ lithium thioglycolate (*p*H 5.1) in 8 $M$ urea solution for paper electrophoresis. The supporting buffer for the electrophoresis was also 0.1 $M$ lithium thioglycolate (*p*H 5.1) in 8 $M$ urea and electrophoresis was carried out using a potential of 120 volts and a current of 5 milliamperes for 15 hours. The two components were eluted separately from the paper by chromatographic washing with dilute ammonia, and after the solutions had been evaporated to dryness *in vacuo* the residue was dissolved in 5 $N$ HCl and hydrolyzed overnight in a sealed tube. By control experiments with the synthetic material it was shown that carboxy-S-methylcysteine could not be satisfactorily separated from glutamic acid by paper chromatography using either phenol/ammonia or methanol/water/pyridine as developing solvents. However the two compounds can readily be distinguished by the use of iodoplatinate as the spraying agent.[16] (If phenol is used as a solvent this must first be completely removed by washing the paper with peroxide-free ether.)

(c) **The Amino Acid Composition of the Anodic and Cathodic Components of Reduced Insulin.**—The technique given above (b) whilst satisfactory for the detection of carboxy-S-methylcysteine required some modification to give definite evidence for the simultaneous occurrence of a number of amino acids because the hydrolysate contained sufficient salt to distort the chromatograms. The following technique was therefore used. After electrophoresis, elution and taking to dryness in the normal manner, the residue (from 4 mg. of starting material) was dissolved in 10 ml. of water and precipitated by the addition of one drop of glacial acetic acid. It was then centrifuged, washed once, and hydrolyzed overnight. As a result of two-dimensional paper chromatograms on the hydrolysates compared with similar chromatograms of control mixtures of amino acids, it appeared that the anodic component agreed qualitatively in composition with Sanger's formulation of the A chain and the cathodic component with the B chain. The spot corresponding to proline in the case of the B chain was faint but this amino acid is difficult to detect with ninhydrin on paper. By separate one-dimensional chromatograms using butanol–water as solvent it was shown that only the anodic component contained isoleucine.

(d) **Quantitative Estimation of Cysteine and Cystine in Insulin Hydrolysates.**—It has previously been shown that some of the cystine of insulin is destroyed during hydrolysis with 5 $N$ HCl and the use of HCl–HCOOH mixtures to prevent this has been advocated.[10,17] In the present work it was found that the presence of urea during hydrolysis exerted a similar protective effect. However even with the additional precaution of hydrolysis in a stream of $N_2$, this was insufficient to prevent loss of cysteine during hydrolysis, since the sum of cystine and cysteine in a hydrolysate of reduced insulin was always substantially less than the cystine content of untreated insulin. Cystine and cysteine were estimated by the Shinohara method,[18,19] using a Coleman Junior Spectrophotometer to measure optical densities.

Untreated insulin (14.6 mg.) was dissolved in 1 ml. of 8 $M$ urea and 8 ml. of 5 $N$ HCl added. Hydrolysis was carried out in a stream of $N_2$ for 16 hours, the solution made up to 25 ml. and thiol and disulfide estimations carried out. These gave the values 0.0% thiol-S, 3.17% cystine-S (on dry weight basis).

Insulin (20.2 mg.) was dissolved in 0.4 ml. of 0.04 $N$ HCl, reprecipitated with 0.4 ml. of 0.04 $N$ NaOH and 0.2 ml. of 0.5 $M$ lithium thioglycolate solution added immediately. (For reproducible results it is essential that the insulin be precipitated in the amorphous form). Reduction was allowed to proceed for 2 hours and then 40 ml. of acid acetone was added and the reduced insulin centrifuged down and washed with acetone. Finally it was dissolved in 1 ml. of 8 $M$ urea, hydrolyzed, and estimations carried out as above, giving the values 0.64% cysteine-S and 2.17% cystine-S on a dry weight basis. There has thus been a loss of 0.36% S during hydrolysis. It was intended to extend this type of estimation to reduction at higher *p*H values, but under these circumstances the sulfur losses became so high that the data had little significance.

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GATES AND CRELLIN LABORATORIES OF CHEMISTRY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

## The Action of Raney Nickel on Selenium Compounds[1,2]

BY HEINRICH HAUPTMANN AND WOLFGANG FERDINAND WALTER

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In connection with our studies of the action of Raney nickel on sulfur compounds we became interested in the behavior of selenium compounds under identical conditions. Since the hydrogenolytic deselenization of such compounds has been described[3] recently, we wish to report our results which were obtained using degassed Raney nickel,[4] thus excluding hydrogen as far as possible. From Table I it can be seen that diselenides and phenyl selenobenzoate are transformed by Raney nickel degassed at 200° into selenides when heated at 80° but that they, as well as diphenyl selenide, yield biphenyl, when the reaction temperature is raised to 180°; a mixture of both reaction products is obtained at 140°. In absence of Raney nickel the selenium compounds are stable in boiling xylene, and melt without decomposition.

TABLE I

TRANSFORMATION OF SELENIUM COMPOUNDS IN PRESENCE OF RANEY NICKEL DEGASSED AT 200°

| Starting material | Heated at °C. | Hours | Comp. obtained | Yield, % | M.p. or b.p. (mm.), °C. |
|---|---|---|---|---|---|
| Diphenyl diselenide | 80[a] | 15 | Diphenyl selenide | 89 | 130–132 (2)[e] |
| | 140[b] | 14.5 | Diphenyl selenide + | 21[d] | 136–142 (4)[e] |
| | | | biphenyl | 31[d] | 68.5–69.5[f] |
| | 180[c] | 7 | Biphenyl | 72 | 68.9–70[f] |
| Di-*o*-tolyl diselenide | 140[b] | 15 | Di-*o*-tolyl selenide | 67 | 61–61.5[f] |
| Phenyl selenobenzoate | 80[a] | 15 | Diphenyl selenide | 54 | 135–140 (3)[e] |
| | 140[b] | 15 | Diphenyl selenide + | 42[d] | 137–143 (4)[e] |
| | | | biphenyl | 15[d] | 68–69.5[f] |
| | 180[c] | 8.5 | Biphenyl | 51 | 69–70[f] |
| Diphenyl selenide | 180[c] | 8 | Biphenyl | 77 | 69.5–710.5[f] |
| Di-*o*-tolylselenide | 180[c] | 8 | Di-*o*-tolyl | 52 | 249–250[g] |

[a] In benzene. [b] In xylene. [c] Without solvent in a stream of nitrogen. [d] Calculated as recovery of phenyl radicals (see ref. 4b). [e] Identified by transformation into diphenylselenium dibromide, m.p. 134°, dec. about 145°. [f] Identified by mixed m.p. with authentic sample. [g] Identified by oxidation to 2,2′-diphenic acid, m.p. 225–228°.

These selenium compounds behave therefore in presence of degassed Raney nickel in the same manner as do the corresponding sulfur compounds.[4] This is true also for the biphenyl formation, since it has been found that the sulfur compounds also give biphenyl when heated with degassed Raney nickel at 220°.[5] However, selenium is eliminated from the molecule at a lower temperature, since biphenyl formation is completed at 180° and is formed in considerable amounts even in boiling xylene, where diphenyl sulfide is the only product obtained from the sulfur compounds.

It seems that the methyl groups in the *o*-positions make the reaction with the Raney nickel somewhat difficult, since di-*o*-tolyl diselenide yields at 140° only the corresponding selenide and no di-*o*-tolyl. This problem is being investigated on selenium as well as on sulfur compounds.

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### Experimental[6]

The Raney nickel used in all experiments was prepared, dried and degassed at 200° as described in previous communications.[4] Three representative experiments are described.

1. **Reaction of Diphenyl Diselenide at 140° with Raney Nickel.**—To a suspension of 65 g. of degassed Raney nickel in xylene, 5.00 g. of diphenyl diselenide[7] was added and the mixture refluxed for 14.5 hr. with continuous stirring. The nickel was then filtered off and washed several times with hot xylene. The combined xylene solutions were then evaporated and the oily residue fractionated *in vacuo*. At 4 mm. there distilled at 125–130° a colorless product which solidified on cooling and after one recrystallization from alcohol melted at 68.5–69.5°. This m.p. was not altered by admixture of authentic diphenyl; yield 0.766 g. A second fraction distilled at 136–142°. The resulting yellowish liquid (0.795 g.) was dissolved in ether, and under cooling 0.3 g. of bromine in ether was added. The mixture was kept in the ice-box with occasional shaking during the first hour. The reaction product was filtered off and recrystallized from carbon disulfide and yielded 1.04 g. (78%) of red needles, which melted at 134° and decomposed at 145° (indicated for $(C_6H_5)_2SeBr_2$: dec. about 148°[8]).

(1) Paper V of the series "The Action of Raney Nickel on Sulfur Compounds." Presented at the XIV International Congress of Pure and Applied Chemistry, Zurich, July, 1955.

(2) This paper is extracted from a thesis presented to the Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo by Wolfgang Ferdinand Walter in partial fulfillment of the requirements for the degree of "Dr. em Ciências."

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