# Simultaneous Reduction and Mercuration of Disulfide Bond A6–A11 of Insulin by Monovalent Mercury†

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ABSTRACT: Insulin reacts with monovalent mercury ions in 0.5 M aqueous acetic acid. Two atoms of mercury are incorporated per protein molecule; only one atom is, however, retained upon gel filtration of the product. It was shown that the mercurous ions selectively reduce and mercurate the disulfide bridge A6–A11 of the protein, forming an S–Hg–S bond. The insulin–mercury complex which is designated [insulin·Hg] is monomeric, behaves on electrophoresis like the native protein, exhibits full combining power with anti-insulin, and exhibits a circular dichroism (CD) spectrum in the region of peptide absorption which is very similar to that of native insulin. The conformation of the insulin–mercury complex is thus similar to that of the native protein. The possible application of the above reaction for the preparation of heavy atom derivatives of proteins for X-ray studies is discussed.

The effect of the elongation of internal bridges of protein molecules on the conformation and biological activity of the protein has been recently studied in a few cases (Steinberg and Sperling, 1967; Sperling *et al.*, 1969; Arnon and Shapira, 1969; Sperling and Steinberg, 1971; Steiner and Blumberg, 1971). These bridges were lengthened by the insertion of mercury atoms between the sulfur atoms of the disulfide bonds. The S–Hg–S sequence thus formed is linear (Grdenic, 1965) and is about 3 Å longer than the S–S bond which it replaces (Yakel and Hughes, 1954; Pauling, 1960; Bradley and Kunchur, 1965). The insertion of a mercury atom into the disulfide bridge IV–V of bovine pancreatic ribonuclease (Sperling *et al.*, 1969), or into the disulfide bond which bridges residues 43 and 152 of papain (Arnon and Shapira, 1969) had no perceptible effect on the conformation or biological activity of these proteins. Similarly, insertion of mercury atoms into the interchain disulfide bonds of the Fab and Fc fragments of a myeloma protein did not affect its activity as an antibody to any appreciable extent (Steiner and Blumberg, 1971). Furthermore, the conversion of *all four* disulfide bridges of ribonuclease into S–Hg–S bridges did not abolish the enzymic activity of the protein, although the catalytic power was appreciably modified (Sperling and Steinberg, 1971). Physicochemical studies have indicated that the elongation of these internal cross-links may have little effect on the conformation of the protein molecules.

The mercury derivatives of bovine pancreatic ribonuclease (Sperling *et al.*, 1969), papain (Arnon and Shapira, 1969), and myeloma protein Fc fragment (Steiner and Blumberg, 1971) were found to crystallize readily. Since the insertion of mercury atoms into disulfide bonds of proteins does not seem as a rule to markedly change the conformation of the protein molecules, the heavy atom derivatives obtained may be useful in the investigation of the structure of proteins by X-ray crystallography. Indeed, the mercury derivative of ribonuclease was shown to yield crystals which were isomorphous with those of the corresponding native protein.

All the above mentioned mercury–protein derivatives were prepared in two steps: reduction of disulfide bonds of the protein (usually by sulfhydryl reagents) and subsequent reaction of the resulting sulfhydryl groups with mercuric ions. Being a two-step reaction this procedure is somewhat cumbersome and suffers from drawbacks in cases where the free sulfhydryl groups formed are unstable and reoxidize readily (Sperling *et al.* 1969). In the present work we applied a new reaction for the simultaneous reduction and mercuration of disulfide bonds in a single step, which overcomes the above difficulties. The reagent used is monovalent mercurous ions, which presumably react with disulfide bonds according to eq 1. (M. M. David, R. Sperling, and I. Z. Steinberg, submitted for publication).

$$Hg_2^{2+} + RSSR \rightleftarrows 2(RSHg)^+ \rightleftarrows RSHgSR + Hg^{2+} \quad (1)$$

The above reaction proved to be of additional advantage in the mercuration of the disulfide bonds of insulin: under proper conditions one disulfide bond was selectively reduced and mercurated. In the following we describe the procedure of the preparation of the monomercury–insulin derivative, the identification of the disulfide bond which was elongated, and some properties of the monomercury–insulin derivative obtained.

## Experimental Section

*Materials.* Crystalline, bovine pancreatic insulin (lot. No. 117B-2580) was purchased from Sigma (St. Louis, Mo.). Twice crystallized pepsin (lot. No. PM691) was obtained from Worthington Biochemical Corporation (Freehold N. J.). Insulin immunoassay kit (Code IM. 39), [$^{203}$Hg]mercuric acetate (MBS 2), and [2-$^{14}$C]iodoacetic acid (lot. No. B14 (−20)) were purchased from the Radiochemical Centre (Amersham, England). All other chemicals were of analytical grade.

*Methods. Spectrophotometric measurements* were made with a Zeiss spectrophotometer, Model PMQ II. Quartz cells of 1-cm light path were used.

*pH measurements* were performed with a Radiometer automatic titrator, Type TTT1C (Radiometer, Copenhagen). The electrodes were a G2222B gass electrode and a K4112 calomel electrode (Radiometer).

*Circular dichroism (CD) spectra* were obtained with a Cary spectropolarimeter, Model 6001. Cells of 10- and 1-mm light path were used. The protein concentration in these studies was approximately 0.1%. CD data are presented as $\Delta\epsilon$, the

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difference between the molar extinction coefficients for left- and right-handed circularly polarized light. Signal to noise ratios at band maxima were about 25:1.

*Ultracentrifugation.* The weight average molecular weights of the protein derivatives were determined by the Yphantis midpoint method (Yphantis, 1960) in a Spinco Model E analytical ultracentrifuge.

*Amino acid analyses* of protein samples were performed with the Beckman-Spinco automatic amino acid analyzer, Model 120C, after hydrolysis in constant-boiling HCl for 22 hr at 110°.

*Radioactivity measurements* were performed with a Packard Model 3003 TriCarb liquid scintillation spectrometer and a Vanguard Model 880 automatic chromatograph scanner. A Packard Model 320E TriCarb flow monitor, attached to the amino acid analyzer, was used for the detection of labeled amino acids. Radioactive peptides obtained after peptide mapping were detected on paper by autoradiography with Kodak BB-54 X-ray film. Exposure time was 12–24 hr.

*Acrylamide disc electrophoreses* were carried out with a Shandon apparatus, using 15% gels, at pH 4.3, for 90 min according to Reisfeld *et al.* (1962).

*Dialysis experiments* were performed with Visking seamless cellulose tubing (Union Carbide Inc.), preheated at 80° for 3 days.

*Electrophoresis on cellulose acetate* membranes was performed using Beckman's Model R-100 microzone electrophoresis system.

*Insulin radio immunoassay* was performed using the Radiochemical Centre insulin immunoassay kit. The assay is based on the competition of insulin in the sample to be assayed with [$^{125}$I]insulin in the assay mixture for reaction with an antibody specific to insulin, by use of the double antibody method of Hales and Randle (1963).

*Pepsin Digestion.* Protein samples (5 mg/ml) were digested with pepsin (2.5% w/w of protein) in 5.0% formic acid for 20 hr at 37°. In all cases the peptic digests were applied to the paper immediately after digestion.

*High-Voltage Paper Electrophoresis.* Material was applied to Whatman No. 3MM paper and subjected to electrophoresis at 60 V/cm for 1 hr at pH 6.5 or pH 1.9 (Katz *et al.*, 1959). After electrophoresis, the paper was dried in a stream of cold air. Longitudinal strips were cut from the paper and developed with ninhydrin–cadmium reagent (Dreyer and Bynum, 1967) and nitroprusside–cyanide reagent (Toennies and Kolb, 1951). The strips were then examined for the location of radioactive spots.

*Paper chromatography* was performed on Whatman No. 3MM chromatography paper. The paper was subjected to descending chromatography in 1-butanol–acetic acid–water (25:6:25 v/v), upper phase, for 24 hr and dried in a stream of cold air.

*Insulin concentrations* were routinely determined spectrophotometrically at 280 mμ using $\epsilon_{280}$ 5500 l. mol$^{-1}$ cm$^{-1}$. This value for $\epsilon_{280}$ was determined from measurements of solutions of known protein concentration determined by nitrogen content by the Kjeldahl–Nessler procedure. The nitrogen content of insulin is 15.88% (Leitch, 1948).

*Mercury Reaction Mixture.* A solution of $10^{-4}$ M $^{203}$Hg(OAc)$_2$ and of $10^{-4}$ N $^{203}$Hg$_2$(OAc)$_2$ was used for mercuration. The function of the bivalent mercury was to stabilize the monovalent mercury and to compete with the monovalent mercury for interaction with the amino groups of the protein. Interaction of the latter with amino groups may result in the liberation of metallic mercury. The mercury reaction mixture was kept in a dark tightly closed bottle to avoid its photodecomposition.

*Exchange of the Mercury with Iodoacetic Acid.* The procedure described earlier (Burstein and Sperling, 1970) was followed: 4 μmol of the mercury–insulin derivative was dissolved in 10 ml of 8 M urea and 1 M Tris-Cl buffer (pH 8.0); 40 μmol of [2-$^{14}$C]ICH$_2$COOH and 100 μmol of EDTA were added, and the reaction mixture was kept under nitrogen for 60 min with constant stirring. The urea and excess iodoacetic acid were removed by ultrafiltration by the Diaflo technique, using UM-2 ultrafiltration membranes. The protein derivative was washed three times with 0.1 M aqueous Tris buffer (pH 8.0) in a 50-ml ultrafiltration cell.

*Reduction of CM-Insulin.* To a solution of 4 μmol of an insulin derivative in 5 ml of 8 M urea–0.1 M Tris-Cl buffer (pH 8.0), 0.5 mmol of β-mercaptoethanol was added. The reaction was kept under nitrogen for 4 hr.

*Aminoethylation.* Ethylenimine (20-fold molar excess per sulfhydryl groups) was added to the stirred reduction mixture of the protein and the reaction was carried out under nitrogen for 30 min. The reaction mixture was purified by gel filtration on a Sephadex G-25 column and eluted with $10^{-3}$ M $NH_4OH$. The fraction containing the A and B chains was lyophilized.

*Peptide Mapping of Tryptic Digest.* The mixture of aminoethylated A and B chains was dissolved in water and 0.5 M phosphate buffer (pH 8.0) was added till the final buffer concentration was 0.1 M. The final concentration of the peptides was 1 mg/ml. Tos-PheCH$_2$Cl[1] treated trypsin (Carpenter, 1967) was dissolved in 0.02 M $CaCl_2$ at a concentration of 2 mg/ml. Aliquots of this enzyme solution were added to the substrate solution to give an enzyme–substrate ratio of 1:100 by weight. Digestion was carried out at 25° for 4 hr. The tryptic digest was applied to Whatman No. 3MM paper (approximately 1–1.5 mg/cm) and subjected to electrophoresis at pH 6.5 at 60 V/cm for 45 min (Katz *et al.*, 1959). The paper was then dried in a stream of cold air and the radioactive peptides were located by autoradiography with Kodak BB-54 X-ray film for 24 hr. The film was developed and the radioactive regions were cut out of the remaining paper and stitched to another piece of Whatman No. 3MM paper. The paper was subjected to electrophoresis at 60 V/cm for 60 min at pH 1.9. After drying, mapping of radioactivity, and staining with ninhydrin–cadium reagent, the regions which were both ninhydrin positive and radioactive were eluted by water, hydrolyzed, and subjected to amino acid analysis.

## Results

*Insertion of Mercury into Insulin.* The extent of mercuration of the disulfide bonds of insulin by monovalent mercury was studied as a function of the reaction time and of mercury concentration. This was investigated by use of $^{203}$Hg in the technique of equilibrium dialysis. The reaction mixture contained 0.1 mg/ml of insulin, $10^{-4}$ M monovalent mercury, and $10^{-4}$ M bivalent mercury in 0.5 M aqueous acetic acid. The mixture was placed in a dialysis bag suspended in a solution of $10^{-4}$ N $Hg_2^{2+}$ + $10^{-4}$ M $Hg^{2+}$ in 0.5 M acetic acid. The excess of mercury in the dialysis bag measures the binding of mercury to the protein.

Figure 1 describes the extent of mercuration of the protein with time, as measured by the above technique. For com-

[1] Abbreviations used are: CM-cysteine, carboxymethylcysteine; $(GS)_2Hg$, oxidized glutathione derivative having an S–Hg–S bridge; Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

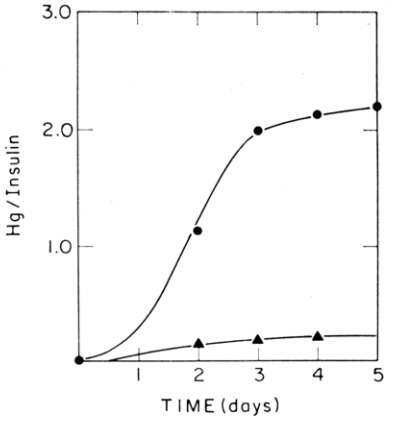


FIGURE 1: Rate of binding of $^{203}Hg$ to insulin measured by equilibrium dialysis: (●) 0.2 mg of insulin + $10^{-4}$ N $Hg_2^{2+}$ + $10^{-4}$ M $Hg^{2+}$ in 2 ml of 0.5 M acetic acid, dialyzed against 1 l. of 0.5 M aqueous acetic acid containing $10^{-4}$ N $Hg_2^{2+}$ + $10^{-4}$ M $Hg^{2+}$; (▲) 0.2 mg of insulin + $10^{-4}$ M mercuric acetate in 2 ml of 0.5 M acetic acid dialyzed against 1 l. of 0.5 M acetic acid containing $10^{-4}$ M mercuric acetate.

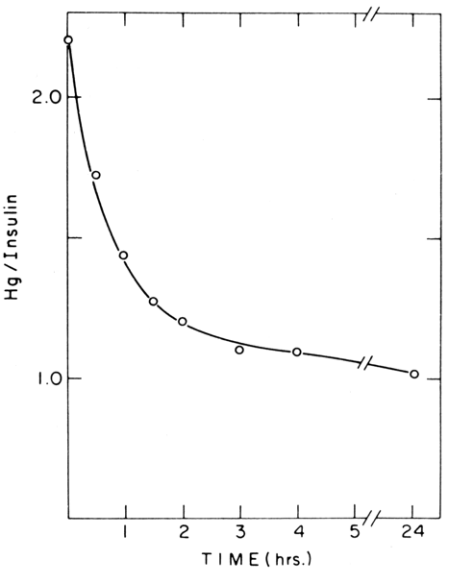


FIGURE 2: Rate of escape of $^{203}Hg$ from a solution of [insulin·2Hg]. Samples of [insulin·2Hg] (0.4 mg in 2 ml of 0.5 M acetic acid) were dialyzed in preheated cellulose tubing against 6 l. of distilled water at pH 7.0 (adjusted by NaOH). Samples were taken at listed intervals and measured for their protein and $^{203}Hg$ content.

parison the extent of binding of mercury to insulin upon reaction of the protein with bivalent mercury under the same conditions is also presented. It may be seen that after 4 days of mercuration by the mercury reaction mixture (*i.e.*, $Hg^+$ + $Hg^{2+}$), two mercury atoms are bound per insulin molecule, while only 0.15 mercury atom are bound per insulin molecule in the absence of monovalent mercury. Longer reaction time (6–8 days) resulted in more extensive incorporation of mercury in both cases. This is probably due to side reactions, as will be discussed below (Ramachandran and Witkop, 1964; Leslie, 1967). The mercuration reaction of insulin was followed by the equilibrium dialysis technique also at other pH values. It was found that after 4 days of reaction 0.5 mercury atom was bound per insulin molecule at pH 6.0 (sodium acetate buffer) and there was no mercuration at pH 8.5 (Tris-Cl buffer). Four days of reaction time in 0.5 M acetic acid were thus chosen for the preparation of the mercury–insulin complexes.

*Preparation of* [*Insulin·Hg*]. Insulin (40 mg) was dissolved in 40 ml of 0.5 M aqueous acetic acid. This mixture was added to 400 ml of an aqueous solution which contained $10^{-4}$ M $^{203}Hg(OAc)_2$ and $10^{-4}$ N $^{203}Hg_2(OAc)_2$, in 0.5 M acetic acid. The reaction mixture was kept in a closed dark bottle for 4 days with constant stirring. After 4 days the reaction mixture was concentrated in a Diaflo ultrafiltration cell using UM-2 membranes. The concentrated solution was passed through a G-25 Sephadex column with 0.5 M acetic acid as eluent. (The Diaflo ultrafiltration cell and the Sephadex column were protected from ambient light with aluminum foil.) The major protein fraction contained one mercury atom per protein molecule. This fraction was collected and designated [insulin·Hg]. A small fraction (about 10% of the total protein) containing two mercury atoms per protein molecule appeared at the front of the column. Dialysis of this last fraction against $H_2O$ at pH 7.0 (NaOH) resulted in loss of mercury and formation of [insulin·Hg] (Figure 2).

*Site of Binding of the Mercury in* [*Insulin·Hg*]. Formation of a mercury mercaptide in the above reaction was established by pepsin digestion of the various mercurated insulin derivatives and high-voltage paper electrophoresis of the fragments formed. Electrophoresis of the digest of native insulin at pH 6.5 resulted in three peptides which contain disulfide bonds, as detected by use of nitroprusside–cyanide reagent (Toennies and Kolb, 1951) (see 1a, Figure 3). Some positive reaction with the nitroprusside–cyanide reagent was obtained at two additional regions: at the origin (not shown in Figure 3), resulting from precipitated protein, and at the neutral-peptide region, resulting from incomplete peptic digestion. Pepsin digestion of [$^{203}Hg$][insulin·Hg] followed by high-voltage paper electrophoresis at pH 6.5 gave the same pattern as the native protein (see 2a, Figure 3). It may be noted that S–Hg–S groups give positive reaction in the cyanide–nitroprusside test. Radioactivity measurements of the electrophorogram revealed only one major peak for the location of $^{203}Hg$, which coincided with one of the peptides reacting positively with the cyanide–nitroprusside reagent (see 2b, Figure 3). Minor radioactive peaks occur at the origin and at the neutral-peptide region, but decrease in intensity with prolonged digestion by pepsin. In the case of mercurated insulin ob-

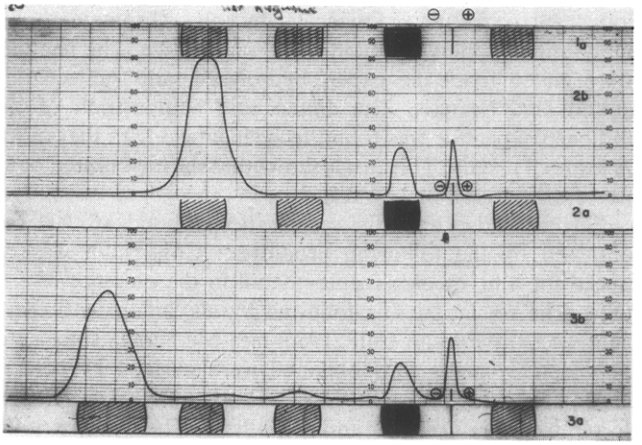


FIGURE 3: Electrophoretic pattern of the peptic digest of insulin derivatives. The digest was fractionated at pH 6.5, 60 V/cm. Strips were cut and (a) developed with ninhydrin and with cyanide–nitroprusside (hatched areas) (spots which reacted with cyanide–nitroprusside were also ninhydrin positive, and *vice versa*), and (b) assayed for their $^{203}Hg$ content. (1) Native insulin; (2) [insulin·Hg]; (3) mercurated insulin after 8 days of mercuration with $Hg_2^{2+}$ + $Hg^{2+}$.

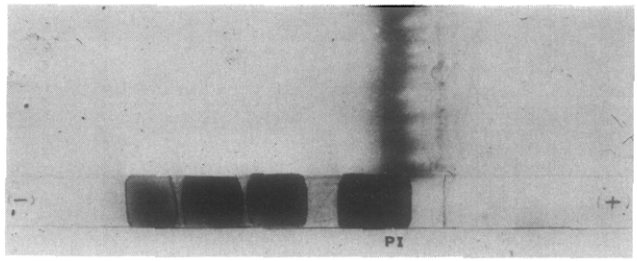


FIGURE 4: Electrophoretic pattern of the tryptic digest of CM-insulin (prepared by exchange of the mercury in [insulin·Hg] with [2-$^{14}$C]-iodoacetic acid) after reduction and aminoethylation. The tryptic digest was fractionated at pH 6.5, 60 V/cm. Strips were cut and developed with ninhydrin–Cd reagent (lower part), and the rest of the elctrophoretogram was autoradiographed for its $^{14}$C radioactivity content (upper part). The radioactivity is concentrated in PI.

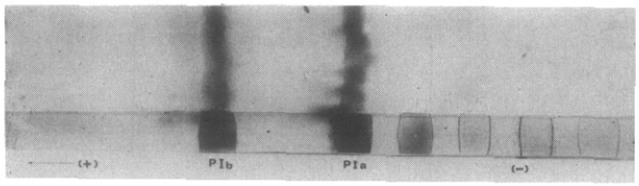


FIGURE 5: Electrophoretic pattern of PI. PI was cut from the paper, stitched to another paper, and subjected to electrophoresis at pH 1.9, 60 V/cm. Strips were cut and developed with ninhydrin–Cd reagent (lower part), and rest of the electrophoretogram was autoradiographed for its $^{14}$C radioactivity content (upper part).

tained upon mercuration for 8 days, the pattern of the pepsin digest obtained upon high-voltage paper electrophoresis differed from that of the native protein (see 3a, Figure 3). This result is probably due to interchanges among the disulfide bonds. Under these circumstances the radioactive mercury appeared in the region of the new disulfide regions (Givol *et al.*, 1965).

Further support for the formation of mercury mercaptides by reacting native insulin with monovalent mercury was obtained by the exchange of the bound mercury with [2-$^{14}$C]-iodoacetic acid and the formation of carboxymethylcysteine, CM-cysteine, residues according to the reaction described by Burstein and Sperling (1970). In the case of [insulin·Hg], 1.1 CM-cysteine was obtained per protein molecule (55% yield of exchange). The carboxymethylated derivative obtained will be called CM-insulin.

*The Identification of the Mercurated Disulfide Bond.* Though the affinity of mercury to sulfhydryl groups is strong, the binding is reversible resulting in loss of mercury during analysis procedures. In order to locate the mercurated bond in insulin this bond was therefore converted to S–C bonds through the formation of [2-$^{14}$C]CM-cysteine derivative (Burstein and Sperling, 1970) (see Methods). The CM-insulin thus obtained was analyzed as follows.

Reduction of [2-$^{14}$C]CM-insulin with β-mercaptoethanol in 8 M urea resulted in splitting of the molecule into the A and B chains. The two chains were carboxymethylated by cold iodoacetic acid and were separated by paper chromatography. Only one peptide was found to be radioactive. The peptides were eluted from the paper, hydrolyzed, and subjected to amino acid analysis. The amino acid composition of the radioactive peptide was found to be identical with that of the A chain of insulin. This result shows that the mercurated bond was the disulfide link A6–A11.

Further evidence for the mercuration of bond A6–A11 was obtained by the following procedure: CM-insulin was reduced for 4 hr by β-mercaptoethanol in the presence of 8 M urea. The reduction was followed by alkylation with ethylenimine (Raftery and Cole, 1966) (see Methods). The insulin polypeptide chains thus obtained were purified by column chromatography on Sephadex G-25 column ($10^{-3}$ M $NH_4OH$) to remove excess reagent and lyophilized. The mixture of the two chains of insulin was then digested by Tos-Phe$CH_2$Cl trypsin, which splits the peptide bonds following lysine, arginine, and aminoethylcysteine, and the digest was subjected to high-voltage paper electrophoresis (see Methods). Figure 4 illustrates the electrophoretic pattern of the tryptic digest. It can be seen that the labeled carboxymethyl group appears in one region, designated PI. PI was subjected to high-voltage paper electrophoresis at pH 1.9 for 60 min (Figure 5). The radioactive region PI separated into a number of ninhydrin-positive spots, only two of which were radioactively labeled, PIa and PIb. These two peptides showed roughly equal radioactive labeling. PIa and PIb were eluted and subjected to acid hydrolysis and amino acid analysis. The results are presented in Table I. Table I also includes the amino acid analysis expected for the peptides A 1–7 and A 8-20 of insulin. It is seen that the amino acid compositions of PIa and PIb are in good agreement with those of A 1–7 and 8–20, respectively. These results show that the peptide bond between the 7th and 8th and between the 20th and 21st amino acids of the A chain of reduced-aminoethylated CM-insulin were split by trypsin, which indicates that Cys-7 and Cys-20 of the A chain were converted into aminoethylcysteine in the above procedure. The low yield of CM-cysteine in PIa can be accounted for by some oxidation of CM-cysteine to carboxymethylcysteine sulfone (Harris, 1967), and by the incomplete substitution of the mercury by the carboxymethyl group. The latter possibility will give rise to peptide A 1–6 with aminoethyl cysteine at A6, which might have a similar mobility to A 1–7 under the conditions used. The above analysis adds proof that cystine A6–A11 (Ryle *et al.*, 1955), which closes the small loop of insulin, was selectively mercurated in the derivative [insulin·Hg].

*Properties of [Insulin·Hg]. Molecular weight* was determined by the equilibrium method of Yphantis (1960). The molecular weight of [insulin·Hg] (in 0.5 M acetic acid–0.1 N NaCl) was found to be 5800 ± 150, similar to that of the native protein. The molecular weight of the protein which comes off the Sephadex column at the front of the peak of [insulin·Hg] was found to be 6990, indicating the presence of some dimers (up to 20%) or higher aggregates.

*Electrophoretic Migration.* [Insulin·Hg] migrated as a single band on acrylamide gel electrophoresis at pH 4.3, and on cellulose acetate at the pH values 4.3, 5.2, and 8.6 (in 8 M urea). In all cases studied the mobility was similar to that of native insulin. The protein coming off at the front of the peak of [insulin·Hg] on the Sephadex column was not homogeneous and contained a slower aggregated fraction beside a fraction which moved like the native protein.

*Binding of Zinc.* [Insulin·Hg] has no zinc bound to it. The Zn was lost during the preparation procedure in acid solution and the passage through the Sephadex column (elution with 0.5 M acetic acid). Native insulin which was incubated in 0.5 M acetic acid for 4 days, concentrated by the Diaflo ultrafiltration cell and passed through the column has also lost its zinc. [Insulin·Hg] can bind Zn, as was shown by use of radioactively labeled Zn and by atomic absorption. This is not surprising as the Zn binding site is not close to the intrachain disulfide bond, as revealed by the three-dimensional structure of insulin obtained by X-ray crystallography (Adams *et al.*, 1969; Blundell *et al.*, 1971).

TABLE I: Amino Acid Composition of the Radioactive Peptides PIa and PIb from the Tryptic Digest of [Insulin·Hg] after Exchange Reduction and Alkylation.

| Amino Acid | PIa ($\mu$mol × $10^2$) | Residues Found[a] | Residues Theor[b] | PIb ($\mu$mol × $10^2$) | Residues Found[a] | Residues Theor[c] |
|---|---|---|---|---|---|---|
| Lysine | | | | | | |
| Histidine | | | | | | |
| Arginine | | | | | | |
| Aminoethylcysteine | 4.08 | 0.9 | 1 | 4.27 | 1.06 | 1 |
| Carboxymethylcysteine | 2.81 | 0.62 | 1 | 3.39 | 0.84 | 1 |
| Aspartic acid | | | | 4.33 | 1.05 | 1 |
| Serine | | | | 7.03 | 1.7 | 2 |
| Glutamic acid | 9.08 | 2 | 2 | 8.26 | 2 | 2 |
| Glycine | 5.43 | 1.2 | 1 | | | |
| Alanine | | | | 4.17 | 1.01 | 1 |
| Valine | 3.86 | 0.85 | 1 | 4.01 | 0.97 | 1 |
| Isoleucine | 3.04 | 0.67 | 1 | | | |
| Leucine | | | | 7.85 | 1.9 | 2 |
| Tyrosine | | | | 5.79 | 1.4 | 2 |

[a] Based on two residues of glutamic acid. [b] Expected for peptide A 1-7 according to Ryle *et al.* (1955). [c] Expected for peptide A 8-20 according to Ryle *et al.* (1955).

*Immunological activity* was monitored by the radioimmunoassay method, which is based on competition of the protein in the sample with iodinated insulin [$^{125}$I] for binding to an antibody which is specific to insulin. The antibody binding activity of [insulin·Hg] was found to be 100–120% of that of the native protein. The front of the peak of [insulin·Hg] eluted off the Sephadex column, which contained two mercury atoms per protein molecule, showed only 60% antibody binding activity, whereas derivatives which were prepared by mercuration for 6–8 days exhibited an even lower activity (10–20%). An insulin derivative which was prepared by 4 days incubation with bivalent mercury and had 0.1 Hg/protein was 100% active, whereas longer incubation times with bivalent mercury for 6–8 days resulted in an incorporation of 1 Hg/protein and has 20% antibody binding activity. It may thus be concluded that reaction for 6–8 days under the conditions described leads to quite drastic changes in the molecular structure, probably by causing interchange reactions among disulfide bonds and other side reactions.

*The ultraviolet absorption spectrum* of [insulin·Hg] is presented in Figure 6. For comparison the absorption spectrum of the native protein is also presented. The absorption spectrum of [insulin·Hg] is slightly different from that of the native protein. This difference may be accounted for by the difference in absorption between the S–Hg–S bridge in the modified protein and that of the S–S group which it replaces. The dots in Figure 6 represent a calculated spectrum obtained by adding the difference between the absorption spectrum of GS–Hg–SG and GS–SG (R. Sperling and I. Z. Steinberg, in prepration) to that of insulin.

*CD Spectrum.* Figure 7 shows the CD spectra of [insulin·Hg] in 0.5 M acetic acid and in 8 M urea–0.1 M Tris buffer (pH

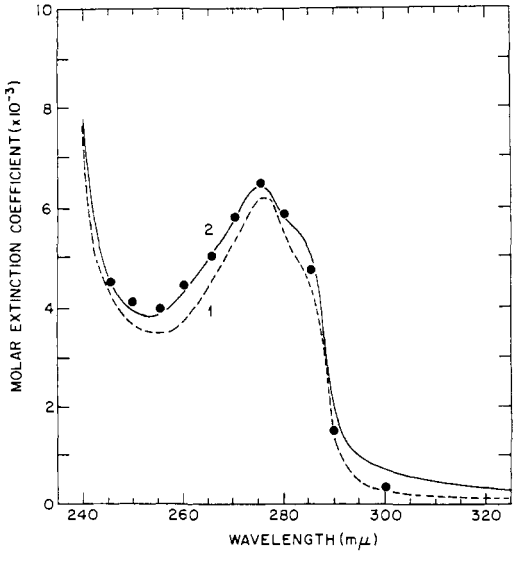


FIGURE 6: Ultraviolet absorption spectrum of insulin (curve 1) and [insulin·Hg] (curve 2); (●) the calculated absorption spectrum of [insulin·Hg] in which the contribution of the S–Hg–S bond to the absorption spectrum has been accounted for from data on model compounds (see text). Measurements were performed in 0.5 M acetic acid.

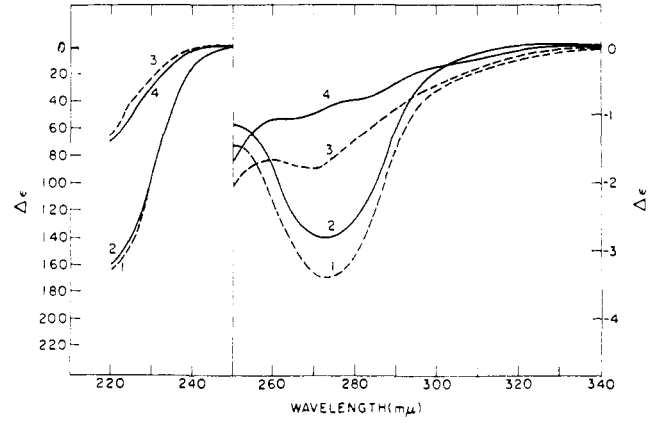


FIGURE 7: Circular dichroic spectra of insulin derivatives: (1) native insulin in 0.5 M acetic acid; (2) [insulin·Hg] in 0.5 M acetic acid; (3) native insulin in 8 M urea–0.1 M Tris buffer (pH 8.0); (4) [insulin·Hg] in 8 M urea–0.1 M Tris buffer (pH 8.0). The spectra are presented as $\Delta\epsilon = \epsilon_l - \epsilon_r$, where $\epsilon_l$ and $\epsilon_r$ are the molar extinction coefficients for left and right circularly polarized light, respectively. (The molecular weight of insulin is taken as 5730, the formula weight.)

8.0). For comparison the CD spectra of native insulin in these two solvents is included. The data for the native protein are in good agreement with those published previously (Beychok, 1965; Morris *et al.*, 1968; Ettinger and Timasheff, 1971). It can be seen that the Cotton effect of [insulin·Hg] in the 250–300-mμ region is lower than that of the native protein, whereas the Cotton effect at 200 mμ is quite the same for insulin and [insulin·Hg]. The pronounced change in the CD spectrum in this region upon the replacement of an S–S chromophore by an S–Hg–S chromophore is not surprising, as studies of low molecular weight model compounds gave a marked CD change in the near-uv region upon converting an S–S bond into an S–Hg–S bond. The fact that the CD spectrum in the region of the peptide absorption bands remain unchanged upon the introduction of the mercury atom into insulin indicates that no gross changes in the conformation of the protein molecule seem to have occurred upon the elongation of the intrachain disulfide bond. Though the CD spectra of insulin and [insulin·Hg] are dramatically decreased upon denaturation (8 M urea, pH 8.0), the *change* in $\Delta\epsilon$ upon denaturation is of comparable magnitude for the two proteins. Since it is rather unlikely that urea would affect the CD spectra of S–S bonds and S–Hg–S bonds to equal extents, one has to assume that urea apparently affects the optical activity of chromophores other than the intrachain bridge in both the native and mercurated insulin.

## Discussion

Mercurous ions react with insulin yielding, under specified conditions, a monomercurated insulin derivative in which a mercury atom has been selectively inserted between the sulfur atoms of the intrachain disulfide bond A6–A11. The mercury–insulin complex resembles native insulin by a variety of criteria: it is monomeric, behaves on electrophoresis like the native protein, retains full combining power with anti-insulin, and exhibit a CD spectrum similar to that of native insulin in the region of peptide absorption. Some differences between the native and modified proteins in the absorption and CD spectra in the range of 240–320 nm have been observed, but can be accounted for by the local alteration at, or near, the modified disulfide bond.

The overall reaction between mercurous ions and disulfide bonds, as presented stoichiometrically by eq 1, formally involves simultaneous reduction of the R–S–S–R bond and mercuration of the R–S groups formed. The reduction of a disulfide bond by mercurous ions yielding sulfhydryl groups and mercuric ions can be shown to be very unfavorable thermodynamically (M. M. David, R. Sperling, and I. Z. Steinberg, submitted for publication). However, the high affinity of the mercuric ions for the sulfhydryl groups to form a mercury mercaptide helps to make the overall reaction described by eq 1 marginally feasible (M. M. David, R. Sperling, and I. Z. Steinberg, submitted for publication; Webb, 1966a–c; Clark, 1960; Edelhoch *et al.*, 1953; Edsall *et al.*, 1954). The fact that the reaction proceeds practically to completion in the case of bond A6–A11 of insulin indicates that the circumstances are specially favorable for the overall reaction in this case. These special circumstances may be responsible for the remarkable selectivity of the reaction of mercurous ions with bond A6–A11 of insulin. The lack of reaction at pH 6.0 and 8.5 seems to be due to the dependence of the free energy of the reaction on pH and buffer used. Kinetic factor cannot, however, be ruled out as determining the observed specificity of the reduction of bond A6–A11. Studies on the mercuration of disulfide bonds of low molecular weight model compounds by monovalent mercury (M. M. David, R. Sperling, and I. Z. Steinberg, submitted for publication) have shown that this reaction is preceded by fast formation of a complex between the disulfide bond and the mercury. Presence of amino groups vicinal to the disulfide bond enhances the stability of the complex, possibly by additional coordination of the mercury to the amino group, and greatly enhances the rate of insertion of the mercury between the sulfur atoms. It is thus conceivable that bond A6–A11 of insulin is mercurated in preference to the other disulfide bonds because of the presence of neighboring amino or amido groups. The amido groups of A-Gln-5 and A-Gln-15 are likely candidates for this purpose (Adams *et al.*, 1969; Blundell *et al.*, 1971).

The reduction of the disulfide bonds in insulin was studied in much detail. Cecil and Weitzman (1964) showed that mild electrolytic reduction results in the splitting of the interchain disulfide bonds; all three disulfide bonds were, however, reduced upon electrolytic reduction at high voltage. Similar results were reported by Markus (1964). Thioglycolate, in the absence of urea, was found to reduce specifically the intrachain disulfide bond (Lindley, 1955). Sodium sulfite was found to reduce only the interchain disulfide bonds; complete reduction was, however, observed in the presence of urea, quanidine hydrochloride, or phenylmercuric hydroxide (Cecil and Loening, 1960). The relative susceptibility of the various disulfide bonds in insulin to reduction thus obviously depends on the details of the reduction procedure. Disulfide bond A6–A11 of insulin is not exposed to the surface of the molecule (Adams *et al.*, 1969; Blundell *et al.*, 1971) and usually is less susceptible for reduction (Blundell *et al.*, 1972). In case of reduction with monovalent mercury the accessibility of the disulfide bond to the surface of the molecule seems to be of less importance. Being a small reagent it can probably penetrate into the molecule.

Insertion of mercury between the sulfur atoms of disulfide bonds in proteins has been previously affected in two steps: reduction of the disulfide bonds by sulfhydryl reagents, followed by reacting the newly formed sulfhydryl groups with bivalent mercury. The method used in the present study presents some obvious advantages. It has proved to be selective for one disulfide bond in insulin. Furthermore, it proceeds under mild conditions. It may be of special advantages in cases where the sulfhydryl groups formed from the disulfide bonds by reduction are labile and reoxidize spontaneously if unprotected (Sperling *et al.*, 1969).

As shown in Figure 1, two mercury atoms are initially bound per insulin molecule upon the reduction of disulfide bond A6–A11. It is pertinent to note that two mercury atoms have been shown to similarly bind to pancreatic ribonuclease in which the disulfide bond IV–V, closing the small loop, has been reduced. In both cases, one of the mercury atoms can be removed, the sequence of reactions being probably as described by eq 1.

Introduction of a mercury atom into a disulfide bond in a protein elongates the internal cross-link by about 3 Å (Yakel and Hughes, 1954; Pauling, 1960; Bradley and Kunchur, 1965). Insertion of a mercury atom between the sulfur atoms of the disulfide bond of oxytocin was previously shown to almost abolish its activity as a uterus-contracting agent (Sperling and Gorecki, 1973). As shown above, this change in dimension of the A6–A11 disulfide bridge of insulin does not seem to have a pronounced effect on the conformation of the protein. Other proteins were also shown to retain their conformation upon similar modification (Sperling *et al.*,

1969; Arnon and Shapira, 1969; Steiner and Blumberg, 1971). The disulfide bond in oxytocin was shown not to be essential to the biological activity of this hormone (Rudinger and Jost, 1964; Jost and Rudinger, 1967). The impairment of the hormonal activity caused by insertion of mercury between the sulfur atoms is therefore most likely to be due to the sensitivity of the conformation of the small polypeptide chain to this modification of the disulfide bridge. Similar elongation of internal bridges which close other loops of equal size in proteins, *i.e.*, in insulin and pancreatic ribonuclease, does not seem to affect the overall molecular conformation. Possibly the rest of the molecule in the larger proteins helps retain the native conformation.

Pancreatic ribonuclease containing a mercury atom between the sulfur atoms of the IV–V bond was shown to crystallize isomorphously with the native protein (Sperling *et al.*, 1969). Attempts to crystallize [insulin·Hg] have as yet been unsuccessful. However, the mercuration reaction of disulfide bonds by monovalent mercury ions is a one-step reaction and proceeds under mild conditions; the possibility thus exists that the above reaction may proceed directly with protein crystals by soaking with a suitable solution containing monovalent mercury. Attempts in this direction may prove to be rewarding for the preparation of heavy atom derivatives of proteins suitable for their study by X-ray crystallography.

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