# Preparation and Properties of Crystals of a Bence-Jones Dimer with Mercury Inserted into the Interchain Disulfide Bond†

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ABSTRACT: A mercury atom was inserted between the sulfur atoms in the interchain disulfide bond of a Bence-Jones λ-chain dimer. The S–Hg–S derivative was prepared both in solution and in crystals by reduction with 2-mercaptoethanol and subsequent reaction with mercuric acetate. Samples of derivative prepared in solution gave crystals with the same space group and lattice dimensions as the native protein, but required less ammonium sulfate for crystallization. Crystals of the derivatives were isomorphous with those of the native protein and were used in the crystallographic study of the dimer. The crystals were less susceptible to radiation damage than other mercurial derivatives or the native protein. The Bence-Jones dimer is represented by two modules of electron density differing in size and structure. The mercury atom in the S–Hg–S derivative was located at the junction between the small module of one dimer and the large module of an adjacent dimer. The determination of the course of each polypeptide chain indicated that the mercury position was associated with the small module. Since the interchain disulfide bond links the penultimate residues, the small module corresponds to the carboxyl halves of the dimer.

One of the major problems in determining the structure of a protein molecule by X-ray crystallography is the preparation of isomorphous heavy-atom derivatives. The insertion of mercury into *inter*chain disulfide bonds to prepare useful derivatives of immunoglobulins and their fragments was suggested by Steiner and Blumberg (1971).

Immunoglobulins of the most prevalent type, IgG, consist of two light (mol wt 22,000–23,000) and two heavy chains (mol wt 50,000–55,000) linked by interchain disulfide bonds. An Fab (antigen binding) fragment released by limited proteolysis of an IgG protein is composed of an intact light chain connected to the amino half (Fd) of a heavy chain by a disulfide bond. The Fc (crystalline) fragment in the enzymatic digest represents a covalently linked dimer of the carboxyl halves of two heavy chains. Monoclonal immunoglobulins suitable for structural work can be isolated in large quantities from patients with multiple myeloma. In some patients the synthesis of heavy and light chains is unbalanced, and the excess light chains appear in the urine, where they are called Bence-Jones proteins.

Under nondissociating conditions the intrachain disulfide bonds in intact IgG proteins or their components are generally not accessible for reduction and subsequent reaction with heavy-metal ions or alkylating agents (Palmer and Nisonoff, 1963; Nisonoff and Dixon, 1964; Steiner and Blumberg, 1971). In the typical case reactivity can therefore be ascribed to the interchain disulfide bonds. Steiner and Blumberg found that the preparation of S–Hg–S derivatives in Fab and Fc fragments was dependent on the number and relative locations of these interchain bonds. For example, in the Fc fragments from the subclass IgG1 there are two closely spaced interchain bonds near the junctions with the Fab regions. The introduction of mercury led to a rearrangement in which half-cystine residues formerly in interchain linkages were incorporated into intrachain S–Hg–S bridges and the dimer was converted into a pair of monomeric subunits held together by noncovalent interactions. Poljak (quoted in Steiner and Blumberg, 1971) found that the crystals of this derivative were not isomorphous with those of the unreacted Fc fragment. With only one interchain disulfide bond, there is no opportunity for such rearrangements in the Fab fragments or in Bence-Jones dimers such as the λ-type molecule (Mcg) described in this article (see also Edmundson *et al.*, 1971, 1972). In λ chains the interchain disulfide bond is formed between the penultimate residues; in members of the second principal antigenic class (κ chains) the bridge is located at the C termini of the two polypeptide chains (Milstein, 1965; Cohen and Milstein, 1967).

## Materials and Methods

*Reaction of the Bence-Jones Protein with Mercuric Acetate in Solution.* The protein was prepared and purified by methods described earlier (Schiffer *et al.*, 1970; Edmundson *et al.*, 1971, 1972). A sample of 124 mg of protein in 1.9 M ammonium sulfate was dialyzed at 4° and at pH 7.4 against 0.02 M Tris-HCl, which had been deaerated and saturated with nitrogen. The solution, now containing 43 mg of protein/ml, was made 0.1 M in 2-mercaptoethanol (Pierce Chemical Co., Rockford, Ill.), covered with a nitrogen blanket, and stored at 22° in a stoppered vessel in the dark for 4 hr. The mercaptoethanol was removed by gel filtration on a 2 × 20 cm column of Sephadex G-25, equilibrated at pH 7.4 with deaerated 0.02 M Tris-HCl. The slurry of Sephadex and buffer had also been deaerated and saturated with nitrogen before the column was poured. The column was shielded from light with aluminum foil, and the buffer was forced through the column under nitrogen pressure. When the absorbance (monitored continuously at 280 nm) began to rise above baseline values, after the emergence of about 15 ml of buffer, the effluent was directed to a tube containing 1.2 ml of 0.01 M mercuric acetate in deaerated Tris buffer. A nitrogen atmosphere was maintained in the receiving tube. After 9 ml had

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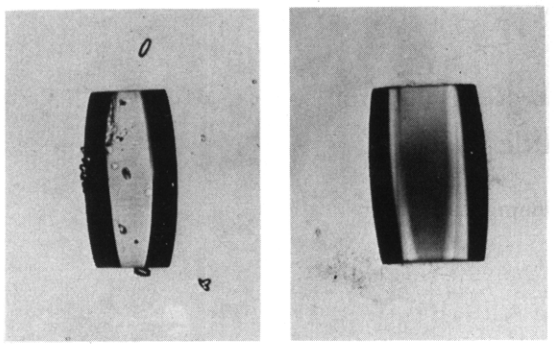

a b

FIGURE 1: Photographs of crystals of the Mcg Bence-Jones protein: (a) native protein crystallized in 1.6 M ammonium sulfate; (b) S–Hg–S derivative (type I) crystallized in 1.3 M ammonium sulfate.

been collected, the absorbance tracing returned to the base line and the remainder of the effluent containing the mercaptoethanol was discarded. The reaction mixture was kept in the dark for 1.5 hr, after which the solution was dialyzed at 4° against the 0.02 M Tris buffer. The dialysis tubing was changed after 8 hr, since Steiner and Blumberg (1971) found that excess mercuric ions were bound by the cellophane membranes. After the change of tubing, the protein solution was dialyzed against 0.02 M phosphate, pH 7.4. If the phosphate was substituted for Tris before the mercuric ions were removed, a fluffy precipitate of mercuric phosphate appeared, with accompanying losses of the S–Hg–S derivative. Prior to crystallization the solution was concentrated by vacuum dialysis to give a protein concentration of about 25 mg/ml. Aliquots containing 3–4 mg of the derivative were made 1.3 M in ammonium sulfate buffered at pH 6.2 with 0.1 M phosphate and stored in vials at 22°. Small crystals were observed within 24 hr, and crystals 1.0–1.5 mm long and 0.4–0.5 mm wide were ready for diffraction experiments in 2–6 weeks.

*Preparation of the S–Hg–S Derivative in Crystals.* The unreacted protein was crystallized in 1.6 M ammonium sulfate buffered at pH 6.2 with 0.1 M phosphate (Edmundson *et al.*, 1972). Four crystals at least 0.45 mm wide were transferred to a vial containing 0.2 ml of a solution (A) consisting of the buffered 1.6 M ammonium sulfate, which had been deaerated and saturated with nitrogen. A 0.05-ml aliquot of a 0.5 M solution of 2-mercaptoethanol in solution A was added to the suspension of crystals (final concentration of mercaptoethanol = 0.1 M). The suspension was covered with a nitrogen blanket, and the stoppered tube was kept in the dark for 4 hr at 22°. The mercaptoethanol solution was rapidly removed by transfer pipet and was replaced by 1.0 ml of solution A. After 1 min 1.0 ml of 0.02 M mercuric acetate in solution A was substituted for the wash solution. The nitrogen atmosphere was reestablished, and the tube was stoppered and stored in the dark for 20 hr. The mercurial solution was gradually replaced by solution A over a period of 1 hr. The crystals were soaked in the latter solution for 3 hr and then mounted in quartz capillaries for X-ray analysis.

*Neutron Activation Analyses.* $\gamma$-Ray spectroscopy subsequent to radioactivation was used to determine the number of gram atoms of mercury in the S–Hg–S derivatives. Each set of type I or type II crystals collectively contained 1–2 mg of protein. The mother liquor over each set was removed and the crystals were washed three times with 0.3 ml of 1.6 M ammonium sulfate, buffered at pH 6.2. The crystals were dissolved in 0.5 ml of deionized water, and the solution was dialyzed exhaustively at 4° against water and lyophilized.

TABLE I: Crystal Data for Native Protein and S–Hg–S Derivatives Prepared in Solution (I) and in Crystals (II).

| | Native | I | II |
|---|---|---|---|
| Space group | $P3_121$ | Same | Same |
| Cell dimensions | | | |
| $a$ (Å) | 72.3 ± 0.2 | 72.4 ± 0.2 | 72.3 ± 0.2 |
| $c$ (Å) | 185.9 ± 0.6 | 186.0 ± 0.6 | 186.1 ± 0.6 |
| Decay (%)[a] | 9–14 | 6–7 | 8–10 |

[a] After exposure at 22° to Cu K$\alpha$ 43 kV, 16 mA. The decay is based on the average decrease in intensity of 30 general reflections from the 6.5-Å shell.

Each sample was dissolved in deionized water, and an aliquot was removed for quantitative amino acid analysis to determine the protein concentration. Aliquots of 10–40 $\mu$l representing 0.4–4.1 $\mu$g of bound mercury in the derivatives were pipetted into 4-mm (i.d.) supracil quartz tubes. In one set of experiments each S–Hg–S solution was made 0.8 M in cesium chloride, used as an internal standard to determine the neutron flux during the radiation; in other samples the internal standard was omitted. The tubes were sealed and irradiated together with tubes containing a range of standards prepared by dilution of a 1000-ppm solution of spectroscopically pure mercuric chloride (ArRo Laboratories, Inc.). Samples consisting of deionized water were used as controls.

The samples were irradiated for 167 hr in the vertical thimble of the 5.0-MW CP-5 heavy water research reactor, with a nominal thermal neutron (0.025 eV) flux of $5 \times 10^{13}$ neutrons/cm$^2$ per sec at peak fission efficiency. The vertical thimble was located on the $^{235}$U fuel core periphery. To lower the background due to nuclides with short half-lives, the samples were not counted for at least 7 days after irradiation.

The detector system was a coaxial drifted diode Ge(Li) crystal (Isotopes, Inc.) with a 1000 channel analyzer. The Ge(Li) crystal had a surface area of 9.6 cm$^2$ and a detector volume of 25 cm$^3$. The crystal resolution measured with the 1333-keV $\gamma$ of $^{60}$Co was 2.7 keV. The analyzer was calibrated with standard sources of $^{88}$V, $^{60}$Co, $^{137}$Cs, and $^{54}$Mn.

*Crystal Data.* A set of intensity data to 3.5 Å (~7500 reflections) was collected in six shells for each derivative on an automated Picker diffractometer, with nickel-filtered Cu K$\alpha$ radiation, by the method described in a previous article (Edmundson *et al.*, 1972). Three crystals, 0.4–0.5 mm wide and cut to a length of 0.4–0.5 mm along $c$, were used to obtain each data set. The crystal data are summarized in Table I.

## Results

*Properties of the Crystals.* Figure 1 shows the similarity in the appearance of the crystals of the S–Hg–S derivative and the native protein. However, the derivative was substantially less soluble than the native protein in ammonium sulfate and required less time to form suitable crystals. The derivative crystals adhered more strongly to the glass vials in which they were grown and also to the walls of the quartz capillaries in which they were mounted for diffraction. This property was advantageous for data collection, during which these crystals moved less in the capillaries than any Bence-Jones crystals we have examined.

TABLE II: Identification of Prominent Peaks in the $\gamma$ Spectrum of the S–Hg–S Derivative of the Bence-Jones Dimer.

| Peak | Isotope[a] | Channel | Energy (keV)[b] |
|---|---|---|---|
| A | $^{203}$Hg | 158 | 279.1 |
| (B) | ($^{134}$Cs,$^{82}$Br) | 310 | (569.2, 554.1) |
| (C) | ($^{134}$Cs,$^{82}$Br) | 331 | (604.6, 618.7) |
| D | $^{137}$Cs | 355 | 661.6 |
| E | $^{82}$Br | 379 | 698.4 |
| F | $^{134}$Cs | 434 | 795.8 |
| G | $^{46}$Sc | 484 | 889.4 |
| (H) | ($^{46}$Sc,$^{65}$Zn) | 608 | (1120.5, 1115) |

[a] Peaks corresponding to more than one isotope are listed in parentheses. [b] Data taken from Adams and Dams (1969).

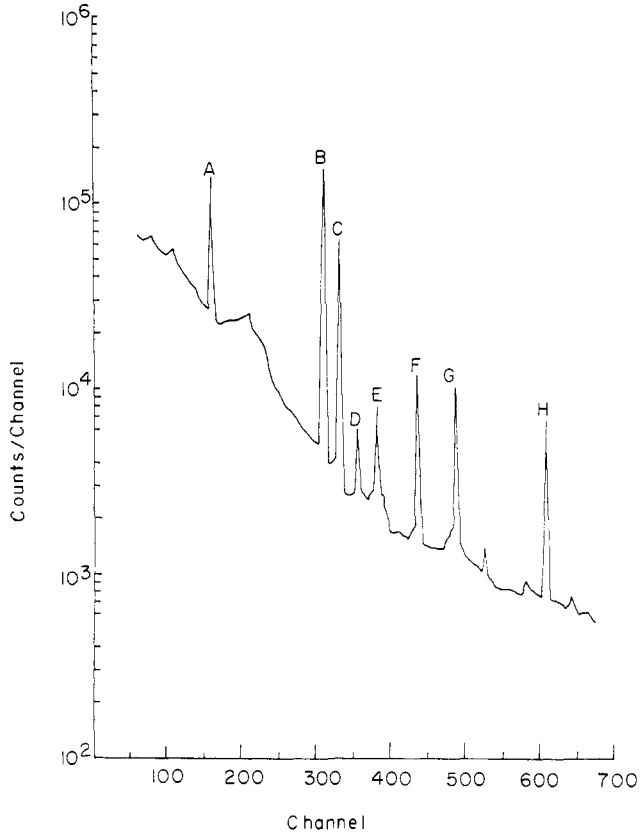


FIGURE 2: $\gamma$-Spectrum of the S–Hg–S derivatives of the Bence-Jones dimer. Crystals of type I were dissolved to obtain the sample. The calculated neutron flux was $8.7 \times 10^{13}$ neutrons/cm$^2$ per sec, the decay period was 7.451 days, and the counting time was 400 min. Peaks are identified in Table II.

Type I crystals (see Table I) were mechanically stronger than those of type II or of other mercurial derivatives prepared by diffusion (*e.g.*, mersalyl sodium, phenylmercuric chloride). Cracks developed in the type II crystals, particularly during the periods when excess reagents were being washed out. The cracks were located mainly along transverse cleavage planes near the centers of the crystals and did not pose serious technical problems.

The presence of the mercury atom in either type I or type II crystals did not change the space group or appreciably alter the unit cell dimensions (see Table I). Furthermore, the S–Hg–S crystals were less susceptible to X-ray damage than any other mercurial derivatives examined (*e.g.*, mersalyl sodium, phenylmercuric chloride, methylmercuric chloride, *o*- and *p*-chloromercuriphenol). The useful crystal life of the S–Hg–S derivative in the X-ray beam was 1.5–2 times that of the other derivatives. Type I crystals appeared to be even more stable than those of the native protein.

*Activation Analyses.* Three representative samples of the S–Hg–S derivatives will be considered: (A) 425 $\mu$g (0.0092 $\mu$mol) prepared by dissolving type I crystals and adding an internal standard of cesium chloride; (B) 898 $\mu$g from type I crystals, devoid of cesium chloride; and (C) 77 $\mu$g from type II crystals, also devoid of cesium chloride. The $\gamma$-ray spectrum for sample A is shown in Figure 2. The characteristics of the prominent peaks are summarized in Table II.

$^{133}$Cs is a 100% abundant isotope and undergoes transformation to $^{134}$Cs when a thermal neutron is absorbed. The capture cross section for this reaction is $28 \times 10^{-24}$ cm$^2$. $^{134}$Cs has a complex decay scheme by $\beta$ emission to an excited state of $^{134}$Ba, with a half-life of 2.05 years. The most prominent $\gamma$ ray accompanying this disintegration is a 795.8-keV $\gamma$ with a branching ratio of 0.88. The effective neutron flux, calculated from data corresponding to the 795.8-keV peak (F) in Figure 2, was $8.7 \times 10^{13}$ neutrons/cm$^2$ per sec (see Kruger, 1971). The activating neutrons were not all of thermal energy, although approximately 90% had energies lower than 0.4 eV. Fortunately, the cross sections of cesium and mercury show similar dependence on energy in the range below 1 eV. However, the presence of even a minor fast flux component increases the importance of including external standards of mercuric chloride among the irradiated samples.

$^{202}$Hg, which has a relative abundance of 29.8%, undergoes the reaction $^{202}$Hg(N,$\gamma$)$^{203}$Hg, with a thermal neutron cross section of $4 \times 10^{-24}$ cm$^2$. The decay of $^{203}$Hg (half-life = 46 days) is accompanied by a 279.1-keV $\gamma$ with a branching ratio of 0.77. On the basis of the calculated neutron flux and the net counts in the 279-keV peak (A) in Figure 2, the amount of mercury in sample A was estimated to be 1.6 $\mu$g (0.0081 $\mu$mol). This corresponds to 0.88 g-atom of mercury per mol of protein dimer.

The calculations for samples B and C were based solely on comparisons with the results for external mercuric chloride standards irradiated under the same conditions. Values of 4.1 and 0.38 $\mu$g of mercury, corresponding to 1.0 and 1.1 g-atoms/mol of protein dimer, were obtained for the two samples. Mercury was not found in the controls.

These analyses indicate no significant differences in the mercury content of type I and II crystals. The results were not markedly affected by the quantities of protein analyzed, at least over a tenfold range beginning with amounts comparable to those in single crystals of the size used in diffraction experiments. The analyses were reproducible when either external or internal standards were employed to compensate for variations in the neutron flux.

Two other elements, zinc and bromine, were detected in the activation analyses of the S–Hg–S samples, but were absent in the controls. In Figure 2, $^{65}$Zn and $^{46}$Sc appear as one peak (H), which was partially resolved into two peaks when a 1600 channel analyzer was used. The recoveries of zinc varied, but ranged as high as 7 g-atoms/mol in different samples. A possible extraneous source of the element is the ammonium sulfate used to crystallize the protein, since zinc is listed as an impurity in the reagent (nominal value $1.4 \times 10^{-6}$ %; Mann Research Laboratories). Preliminary experiments indicate

TABLE III: Final Heavy-Atom Parameters for S–Hg–S Derivatives.[a]

| | $x$ | $y$ | $z$ | $B$ | $A$ | $E$ | $R_K$ (All) | $R_C$ (Centric) | $R_F$ (Centric) |
|---|---|---|---|---|---|---|---|---|---|
| Type I | 0.111 | 0.542 | 0.110 | 2.2 | 269 | 196 | 0.060 | 0.61 | 0.14 at 3.5 Å |
| | | | | | | | 0.069 | 0.54 | 0.18 at 6.5 Å |
| Type II | 0.111 | 0.542 | 0.110 | 3.3 | 278 | 197 | 0.061 | 0.60 | 0.14 at 3.5 Å |
| | | | | | | | 0.055 | 0.46 | 0.17 at 6.5 Å |

[a] $B$ = temperature factor; $A$ = occupancy; $E$ = lack of closure error on an arbitrary scale; $R_K = [\Sigma|F_{PH,o} - F_{PH,c}|]/\Sigma F_{PH,o}$; $R_C = [\Sigma|F_{H,o} - F_{H,c}|]/\Sigma F_{H,o}$; and $R_F = \Sigma|F_{PH} - F_P|/\Sigma F_P$, where $F$ = structure factor amplitude; P = protein; H = heavy atom, and PH = protein plus heavy atom.

that the zinc and bromine are both bound to the protein, but the sites have not been identified.

*Difference Fourier Analyses.* These analyses indicated that the amounts of mercury specifically bound were the same within ±10% in the two types of crystals. In a test of the reproducibility of the diffusion technique, type II crystals prepared 1 month apart were indistinguishable (±1%) in their quantities of specifically bound mercury.

In the absence of prior reduction of the interchain disulfide bond, crystals of native protein did not form derivatives when soaked for 6 weeks in 0.001 M mercuric acetate or chloride.

The position of the mercury atom in types I and II crystals was determined from two- and three-dimensional difference Fourier syntheses, using phases previously obtained for the native protein with other mercurial derivatives. Two-dimensional difference maps for 6.5-Å data are presented in Figure 3. For single-site derivatives three peaks were expected and found in the projections for one-half the unit cell. In three-dimensional difference maps for both 6.5- and 3.5-Å data sets, only one major peak was detected for each asymmetric unit (the Bence-Jones dimer), with no secondary peak being higher than one-fifth the contour level of the main peak.

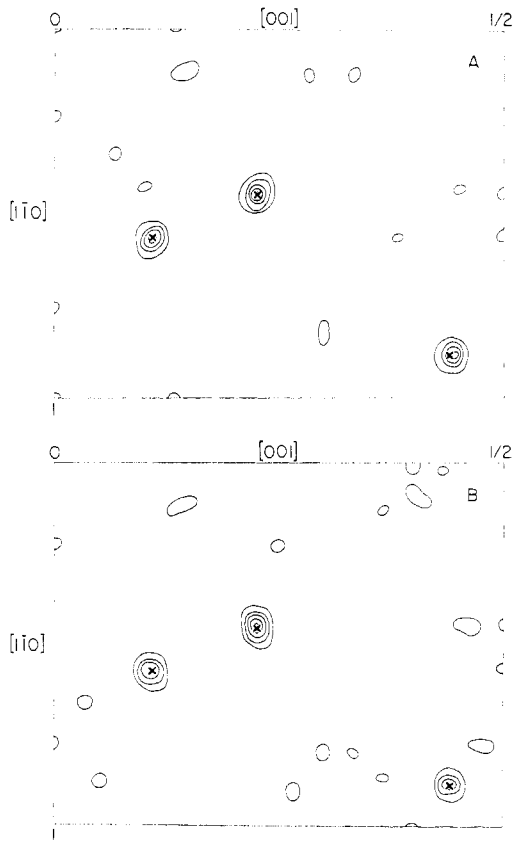


FIGURE 3: Difference Fourier projections for the S–Hg–S derivatives: upper panel, type I; lower panel, type II. Each site is represented by three peaks (designated by ×) corresponding to the three asymmetric units in half the unit cell. The maps are on an arbitrary scale with the contour interval at 200 and the zero contours omitted.

The mercury positions and the isotropic temperature factors were refined together with the data for other mercurial derivatives by a least-squares procedure (Dickerson *et al.*, 1968). The final parameters for the mercury atoms in types I and II crystals are listed in Table III.

The fractional change in the structure factors ($R_F$ in Table III) caused by insertion of the mercury atom was approximately the same in types I and II crystals. At 6.5 Å the mercury atom contributed heavily to the phases. With increasing values of sin $\theta$ (*i.e.*, as the resolution increased), the $R_F$ values gradually decreased and the mercury atom contributed less to the phasing.

The difference in the concentrations of ammonium sulfate in the type I crystals (1.3 M) and those of the native protein (1.6 M) resulted in a "salt effect" at low values of sin $\theta$. The differences in structure factors for the low-order reflections were greater than those between the native and type II crystals, in which the salt concentrations were the same. This salt effect probably accounts for the differences in the average $R_C$ values for the 6.5-Å spheres of types I and II crystals (see Table III).

The three-dimensional 3.5-Å difference Fourier maps showed small positive and negative peaks 5–10% as high as the mercury peak, but some of these represent diffraction ripples (see Brändén, 1964). There were no large negative or positive peaks that might represent displaced sulfur atoms, side chains, or polypeptide backbone in the immediate vicinity of the mercury atom.

The S–Hg–S derivatives were used with other mercurial derivatives to calculate electron density maps of the Bence-Jones dimer at both 6.5 and 3.5 Å. On these maps, which will be discussed at a later date, the mercury atom was located at the junction between the small module of one dimer and the large module of an adjacent dimer. In the 3.5-Å map, the mercury position superimposed on a section of weak electron density, which was near a well-defined ribbon of higher density representing the surface of the larger module. Because of the close packing of the dimers in this region of the crystal, the foregoing information was not sufficient to identify the module containing the C termini. Positive identification was obtained during the recent tracing of the polypeptide chains, in which the carboxyl domains are clearly associated with the smaller module (M. Schiffer *et al.*, manuscript in preparation).

The use of S–Hg–S derivatives in the phasing was expected to result in distortion of the electron density for the interchain disulfide bond in the native protein. The density was substantially lower than that for any of the four intrachain disulfide bonds, an observation which can be partly explained by the lack of complete isomorphism at the mercury site. It may also be attributable to the fact that the exposed interchain bond has more freedom to move than the intrachain inkages, with a resulting decrease in observed density.

## Discussion

Single-site S–Hg–S derivatives of the Mcg λ-chain dimer can be prepared both in solution and in crystals. The two procedures should be applicable to dimers of κ-type light chains, as well as other λ chains or Fab fragments. The terminal interchain disulfide bond in κ chains should be even more accessible to reduction and reaction with mercuric ions than the penultimate disulfide linkage in λ chains.

Performing the required reactions in the crystals is the only procedure which can be used in cases in which the native protein crystallizes, but the S–Hg–S derivative does not. This diffusion method is a highly reproducible microtechnique which takes only 1 day to prepare a crystallographically useful derivative.

The solution method takes longer, but requires less technical skill. It has the advantages of homogeneous reaction conditions and production of derivatives on a larger scale. The solution method is more favorable for preparation of double isomorphous derivatives because the crystals are free of cracks prior to the introduction of a second heavy atom. Both the diffusion and solution methods are 80–100% quantitative with our particular Bence-Jones protein.

The marked decrease in the solubility of the S–Hg–S derivatives in ammonium sulfate and the increase in the affinity of the crystals for glass and quartz are advantageous changes which we cannot presently explain. The enhanced stability in the X-ray beam is also of interest, since unmodified disulfide bonds in some proteins are subject to radiation damage (Patten and Gordy, 1960; Henriksen, 1966; Tolbert, 1966). The bond can be broken, with the formation of RS· free radicals, and subsequent oxidation may occur at the sulfur atoms. The insertion of mercury probably reduces the rate of formation of radicals. The protein is only partially protected, presumably because such features as the intrachain disulfide bonds (inaccessible to the reducing agents and mercuric ions under nondenaturing conditions) also participate in reactions induced by radiation. Damage to proteins in general is not restricted to disulfide bonds, but can also occur at side chains and peptide bonds, with accompanying transfer of energy along the polypeptide chain (Patten and Gordy, 1960; Henriksen, 1966; Tolbert, 1966; Shields and Hamrick, 1970). For example, Blake and Phillips (as quoted in Phillips, 1963) noted that radiation damage resulted in a gradual change from crystalline to amorphous state in myoglobin, which contains no disulfide bonds.

Further studies are required to determine the configuration of the S–Hg–S bond and the type of coordination around the mercury atom. In small molecules these properties are influenced by the bulkiness of the groups attached to the sulfur atoms and by the availability of Lewis bases for coordination with the mercury atom (Aurivillius, 1950; Harding, 1958; Bradley and Kunchur, 1964; Brändén, 1964; Cheung and Sim, 1965; for comparison with normal disulfide bonds, see Steinrauf *et al.*, 1958; Oughton and Harrison, 1959).

In mercury methylmercaptide, for example, the S–Hg–S bond is linear (Bradley and Kunchur, 1964). The mercury–dithizone complex has an S–Hg–S bond angle of 155°, with a nitrogen atom from each aro group weakly coordinating to the mercury atom and helping form a distorted tetrahedron around it (Harding, 1958). An example of a more regular tetrahedral configuration is found in 1,6-dithiacyclodeca-*cis*-3,*cis*-8-dienebis(mercuric chloride), in which the S–Hg–S bond angle is 109° (Cheung and Sim, 1965).

## Acknowledgments

We thank Dr. L. Steiner for her advice and particularly for her suggestion to use 2-mercaptoethanol to reduce the interchain disulfide bonds, Mr. M. D. Fausch for his assistance, and Dr. H. F. Deutsch for his continuing interest and encouragement.

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