A copper-immunoglobulin complex isolated from a myeloma patient with hypercupremia has been investigated in an attempt to localize and characterize the copper-binding site. Cleavage of the purified IgG type immunoglobulin with papain yielded peptides which were identified as Fab and Fκ fragments by immunodiffusion. The fragments were separated by DEAE-cellulose chromatography and the Fab fragment then was purified to homogeneity by chromatography on Sephadex G-100 and protein A-Sepharose. Copper is bound only to the Fab fragment. In contrast, the Fκ fragment, but not the Fab fragment, binds to concanavalin A, suggesting that the carbohydrate moiety on the Fκ fragment is not involved in the binding of copper.

Light and heavy chains were isolated by gel filtration of the products formed upon reaction of the immunoglobulin with mercaptoethanol and iodoacetamide. Acid hydrolysates of light chain, heavy chain, and Fab of the products formed upon reaction of the immunoglobulin with papain yielded peptides which were identified as Fab and Fκ fragments by immunodiffusion. The fragments were separated by DEAE-cellulose chromatography and the Fab fragment then was purified to homogeneity by chromatography on Sephadex G-100 and protein A-Sepharose. Copper is bound only to the Fab fragment. In contrast, the Fκ fragment, but not the Fab fragment, binds to concanavalin A, suggesting that the carbohydrate moiety on the Fκ fragment is not involved in the binding of copper.

The copper-Fab complex, like the copper-IgG complex, is colorless with no detectable visible absorption peak. Dialysis of the copper-Fab against cyanide yields apo-Fab; reconstitution with copper ions yields a complex with 1 copper atom/Fab fragment. The isolated and reconstituted copper-Fab complexes are EPR-nondeTECTable before and after treatment with ferricyanide.

Comparison of the copper-Fab and apo-Fab in terms of fluorescence spectra, circular dichroism spectra, and immunological properties revealed no structural differences due to copper binding. Reaction with dithiodipyridine demonstrates the presence of one exposed sulfhydryl group in apo-Fab but not in isolated or reconstituted copper-Fab complexes. The dithiodipyridine-reacted apo-Fab no longer binds copper, indicating that this sulfhydryl group might be involved in cupper binding. A preliminary experiment indicates that the involved sulfhydryl is labeled upon reaction with [14C]-iodoacetamide.

In 1976, Lewis et al. (1) described a patient with multiple myeloma and hypercupremia. Copper balance and radioisotopic studies suggested a normal gastrointestinal and renal threshold for copper but indicated a specific and tight binding between copper and a large molecular weight serum component. This serum component was shown to migrate with the γ-globulin fraction on cellulose acetate electrophoresis, thus distinguishing it from albumin and ceruloplasmin.

In a subsequent report (2), we described the isolation of this copper-binding component and identified it as an IgGκ (Gm b) type immunoglobulin with 1 light chain. The copper-binding immunoglobulin was shown to be of normal molecular weight (150,000) with normal size light and heavy chains. The copper of the immunoglobulin complex exhibited no EPR signal or visible absorbance spectrum. The immunoglobulin was shown to bind 2 atoms of copper which could be removed by dialysis against cyanide. Incubation of the apo-IgG with copper fully reconstituted the copper-immunoglobulin complex.

In the present report, the copper-immunoglobulin complex is further investigated. We present evidence that 1 atom of EPR-nondetectable copper is located on each Fab fragment and that a thiol group is involved in the binding of copper.

**Experimental Procedures**

**Materials**

4,4'-Dithiodipyridine was purchased from Aldrich Chemical Co.; [14C]iodoacetamide from New England Nuclear; Freund’s adjuvant and agarose agar from Difco Laboratories; rabbit antisera to the Fab fragment, Fκ fragment, and κ light chains of human IgG from Miles Laboratories, Inc.; ovalbumin and chymotrypsinogen A from Worthington; Sephadex G-200 (40 to 120 mesh), concanavalin A-Sepharose, blue dextran, protein A-Sepharose CL-4B, and Sephadex G-25 (50 to 150 mesh) from Pharmacia; 2-mercaptoethanol from Matheson, Coleman and Bell; dialysis tubing and nitrogen (purified grade) from Union Carbide; DEAE-cellulose and sodium lauryl sulfate from Fisher; acrylamide and methylene bisacrylamide from Eastman; Coomassie brilliant blue from Mann Research; bovine serum albumin (Fraction VI), myoglobin, and human γ-globulin from Pentex; Difco ultrafiltration cell and ultrafiltration membranes from Amicon; AG 1-X8 resin from Bio-Rad; and NCS tissue solubilizer from Amersham/Searle.

**General Procedures**

Procedures described previously (2) were used for copper analysis, disc gel electrophoresis in the presence and absence of SDS, chromatography on Sephadex, DEAE-cellulose, and protein A-Sepharose, and UV, visible, and EPR spectral characterization. Molecular weights were determined by SDS disc gel electrophoresis (3) and by gel exclusion chromatography (4) using a Sephadex G 100 column calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome c. Protein concentrations were measured by the procedure of Lowry et al. (6) using bovine serum albumin as a standard or by absorbance at 280 nm using extinction coefficients of 225 μM−1 cm−1 for IgG and 75 μM−1 cm−1 for the Fab fragment (6).

Near UV and far UV CD spectra were measured and recorded at 25°C with a Jasco model ORD/UV-5 optical rotatory dispersion recorder. Fluorescence excitation and emission spectra were measured and recorded at 25°C on a ratio-quantum counter corrected recording spectrophotometer.

1 The abbreviations used are: IgG, immunoglobulin G; SDS, sodium dodecyl sulfate.
Copper-binding Site of a Myeloma Protein

Preparation and Purification of Copper-F_{ab} Fragment

Papain Hydrolysis—Using the procedures outlined by Franklin (8), papain was used to hydrolyze the copper-immunoglobulin complex into its F_{ab} and F_{c} fragments. The copper-immunoglobulin complex in 0.1 M sodium phosphate buffer, pH 6.8, containing a few drops of toluene, was allowed to preincubate in a shaking water bath at 37°C for 30 min. Activated papain was then added to give a papain/copper-immunoglobulin ratio of 1:100, and the sample was incubated at 37°C. After 22 h, the hydrolysate was placed in a precooled dialysis bag and dialyzed against 750 ml of 0.01 M sodium phosphate buffer, pH 8.0, for 26 h. The buffer was changed after the first 24 h.

DEAE-cellulose Chromatography—The dialyzed hydrolysate was fractionated by chromatography on a DEAE-cellulose column (1 X 21 cm) that had been pre-equilibrated with the dialysis buffer. The column was washed with 0.01 M sodium phosphate buffer, pH 8.0, until the absorbance at 280 nm of the eluate was less than 0.02. The column was then eluted with a 0 to 0.4 M linear gradient of sodium chloride in the sodium phosphate buffer (total volume of 140 ml). The column was stripped with 1 M sodium chloride in 0.01 M sodium phosphate, pH 8.0. The elution rate was approximately 10 ml/h.

Sephadex G-100 Chromatography—The copper-peptide fraction which had washed through the DEAE-cellulose column with the equilibrating buffer was pooled, concentrated by ultrafiltration (PM-10 membrane), and further purified by gel filtration on a Sephadex G-100 column. The column (1.8 X 118 cm) was eluted with 0.05 M potassium phosphate buffer, pH 7.2. Approximately 2.5 ml fractions were collected at a flow rate of 10 ml/h.

Preparation and Purification of apo-F_{ab}

Copper was dissociated from the copper-F_{ab} complex by dialysis against cyanide following the same procedure that had been used to remove the copper from the copper-IgG complex (2) except that a 0.1 M concentration of KCN was employed. Gel filtration chromatography on Sephadex G-25 resolved the copper-cyanide complex and the apo-F_{ab}. The isolated apo-F_{ab} was essentially free of copper.

^{14}C Labeling of apo-F_{ab}

The apo-F_{ab} fragment derived from the copper-IgG complex was labeled with [^{14}C]iodoacetamide. Apo-F_{ab} was equilibrated with 0.55 M Tris/phosphate buffer, pH 8.0, and deoxygenated with nitrogen. The solution was made 0.05 M 2-mercaptoethanol, stopped, and stored at -20°C. The solution was then dialyzed against 0.01 M potassium phosphate buffer, pH 8.0, and deoxygenated with nitrogen. The copper solution was added to the apo-F_{ab} solution at a ratio of 1:100, and the sample was allowed to incubate at 0°C for 30 min. The solution was then dialyzed overnight in a precooled dialysis bag against 1000 ml of 0.01 M sodium chloride. The dialysis solution was changed after the first 24 h.

The protein solution was equilibrated with 1 M proionic acid and applied to a Sephadex G-200 column (1.9 X 95 cm). Elution with 1 M proionic acid solution gave three peptide peaks. SDS disc gel electrophoresis indicated that the second peak is the heavy chain derivative, the third peak is the light chain derivative, and the first peak is incompletely reacted immunoglobulin. The third peak was identified as light chain derivative by immunoaffinity against light chain antiserum.

Preparation of Antibodies to the Copper-F_{ab} Fragment

Male New Zealand white rabbits, weighing 3 to 6 kg, were used for immunization. A homogeneous copper-F_{ab} solution (approximately 1.0 ml containing 6 mg) was mixed with an equal volume of complete Freund's adjuvant. The mixture was injected subcutaneously at four or five spots on the upper back of each of two rabbits. One week later, a booster of the same concentration of copper-F_{ab} was injected in the identical manner. Five days following the booster injection, approximately 40 ml of blood was taken from the marginal vein of an ear of each rabbit and allowed to clot. The sera were centrifuged at 2000 X g for 10 min, the supernatant was removed with a pipette.

The antibodies were partially purified from the serum by several ammonium sulfate precipitation steps. Ten milliliters of saturated ammonium sulfate were added to 20 ml of serum. After 10 min at room temperature, the precipitated immunoglobulins were sedimented by centrifugation at 20,000 X g for 15 min. The supernatant fraction was discarded and the pellet resuspended in 10 ml of water. This process of ammonium sulfate precipitation, centrifugation, and resuspension of the pellet was repeated two additional times. The pellet was then resuspended in water and dialyzed extensively against 0.01 M potassium phosphate buffer, pH 8.0.

RESULTS AND DISCUSSION

Localization of the Copper on the F_{ab} Fragment—Incubation of the copper-immunoglobulin complex with the proteolytic enzyme papain resulted in cleavage of the molecule into F_{ab} and F_{c} fragments, as well as smaller peptides. DEAE-cellulose chromatography effectively separated the hydrolysate into three peptide fractions (Fig. 1). All of the copper was associated with the fraction that was washed through the column with the equilibration buffer without binding (Fraction I). A small amount of peptide with no copper (Fraction II) was eluted with a linear gradient (0 to 0.2 M) of NaCl, and a much larger amount of 280 nm absorbing material (Fraction III) was liberated when the column was stripped with buffer containing 1.0 M NaCl.

Electrophoresis on polyacrylamide disc gels showed that the copper-peptide Fraction I (Fig. 2A) and the non-copper-containing Fraction II (Fig. 2C) together accounted for all of the peptide bands exhibited by the total hydrolysate (Fig. 2B). Although each fraction showed multiple bands on disc gels, no cross-contamination between the two fractions could be detected. No peptide bands were detected when Fraction III was incubated with copper(I).
Copper-binding Site of a Myeloma Protein

Fig. 1. DEAE-cellulose chromatography of a papain hydrolysate of the copper-binding immunoglobulin. The arrow at Fraction 42 indicates the initiation of a gradient (0 to 0.4 M) and the arrow at Fraction 125 indicates the addition of 1.0 M sodium chloride to the eluting buffer. Approximately 2.5-ml fractions were collected. The protein fractions were numbered Z, ZZ, and ZZZ in order of their elution from the column. See text for details.

Fig. 2. Polyacrylamide disc gel electrophoresis of the papain hydrolysate fractions from the DEAE-cellulose chromatography (see text for details). The gels were formed and run according to the procedures of Brewer and Ashworth (12). Gels were stained with Coomassie brilliant blue. Fraction I is shown in Gel A and Fraction II is shown in Gel C. The total hydrolysate is shown in Gel B. Fraction III showed no protein bands when stained with Coomassie brilliant blue (gel not shown). All of the protein bands in the total hydrolysate are found in Gels A or C.

III was electrophoresed and stained in polyacrylamide disc gels, suggesting that this fraction probably consists of very small polypeptides.

Whereas the copper peptide (Fraction I) gave negative precipitin tests with concanavalin A, both of the copperless fractions (II and III) gave positive tests, suggesting that they both contain carbohydrate residues.

Sequential chromatography of the copper-fragment on columns of Sephadex G-100 and protein A-Sepharose yielded a fraction that appeared homogeneous. The purified sample showed a single band upon electrophoresis at pH 8.6 on 7.5% polyacrylamide gel (Fig. 3A). Upon electrophoresis on SDS-polycrylamide gels in the presence of 2-mercaptoethanol, this same protein fraction exhibited two peptide bands (Fig. 3B), of approximately 28,000 and 33,000 daltons, respectively.

Identity of the partially purified fragments produced by papain treatment was accomplished by immunodiffusion against anti-human F\(_{ab}\) and anti-human F\(_{c}\) antisera. Fig. 4A shows that a precipitin line of complete identity with the copper-immunoglobulin complex (Wells I and 4) was obtained when the copper-peptide (Fraction I, Well 2) was allowed to diffuse against anti-human F\(_{ab}\) antiserum (center well). No precipitin band was obtained when the copperless Fractions II (Well 3) and III (Well 5) were allowed to diffuse against the same antiserum. Fig. 4B shows that a precipitin line of complete identity with the copper-immunoglobulin complex (Wells I and 4) is observed when Fraction II (Well 3) was allowed to diffuse against anti-human F\(_{c}\) antiserum (center well). Neither the copper-peptide (Fraction I, Well 2) nor Fraction III (Well 5) formed precipitin bands when allowed to diffuse against this same antiserum.

The results of these immunodiffusion tests suggest that the copper-peptide (Fraction I) is the antibody-combining (F\(_{ab}\)) fragment of the copper-binding immunoglobulin. The copperless Fraction II probably constitutes the F\(_{c}\) fragment of the immunoglobulin. Fraction III probably consists of small peptides derived from the F\(_{c}\) fragment of the copper-binding immunoglobulin.

The observation that the F\(_{c}\) fragment formed a precipitate with concanavalin A, whereas the copper-F\(_{ab}\) complex did not,
suggested that the reactive carbohydrate residues were not located on the F\textsubscript{\alpha} fragment and were not involved in the binding of copper. Likewise, the affinity of protein A-Sepharose for the copper-Sepharose complex but not for the copper-IgG complex. Fractions I and III did not form precipitin bands. Diffusion against antisera to the F\textsubscript{\alpha} fragment of normal human IgG. Fraction I formed a precipitin line of complete identity with the copper-IgG complex. Fractions II and III did not form precipitin bands. B, diffusion against antisera to the F\textsubscript{\beta} fragment of normal human IgG. Fraction II formed a precipitin band of complete identity with the copper-Sepharose complex. Fractions I and III did not form precipitin bands.

Characterization of the Copper-F\textsubscript{\alpha} Complex—The molecular weight of the copper-F\textsubscript{\alpha} fragment was estimated from its elution volume from a Sephadex G-100 column. A molecular weight of 50,000 was obtained for this fragment from a standard curve plot of log (molecular weight) versus $V/V_0$. The copper-F\textsubscript{\alpha} complex contained 1.38 atoms of copper/50,000 daltons of protein. The copper-F\textsubscript{\alpha} exhibited two major peptide bands (Fig. 3B) upon electrophoresis on SDS-polyacrylamide gel in the presence of 2-mercaptoethanol. The molecular weights of these peptides were estimated to be 28,000 and 33,000, which presumably correspond to the presence of light chain and the F\textsubscript{\alpha} portion of heavy chain, respectively. The value of 28,000 is the same value obtained for the light chain by electrophoresis of the copper-immunoglobulin in the presence of SDS and mercaptoethanol (2). A minor protein band of approximately 17,000 daltons was seen on overloaded gels. This band was not observed when homogeneous light chain or copper-immunoglobulin were electrophoresed in this system and was presumed to be a degradation product of the F\textsubscript{\alpha} fragment of the heavy chain.

The UV and visible absorption spectra of the copper-F\textsubscript{\alpha} fragment are shown in Fig. 5A. No absorption peak was detected in the visible region of the spectrum at the 80.8 $\mu$m concentration employed. Under these conditions, absorption with a molar extinction of 60 would have been detectable. It should be pointed out that a number of copper complexes have absorptivities less than this in the wavelength range reported here. The UV spectrum showed a single sharp absorption peak at 280 nm.

The UV CD spectra of the copper-F\textsubscript{\alpha} fragment are shown in Fig. 6A. The far UV spectrum showed a large negative Cotton effect at 218 nm. Negative Cotton effects near this region as well as a positive band at 200 nm have been shown to be indicative of $\beta$ structure (13–15). In the near UV region, a positive Cotton effect was observed at 285 and 290 nm. Although the effects of other aromatic side chains cannot be fully excluded, bands near this region have been attributed to tryptophan (16, 17).

The fluorescence excitation spectrum of the copper-F\textsubscript{\alpha} fragment (Fig. 7A), obtained by monitoring the fluorescence at 320 nm while scanning the excitation spectrum, showed the maximum excitation at 275 nm. Both tyrosine and tryptophan show excitation maxima at this wavelength (18). The fluorescence emission spectrum, obtained by exciting the copper-F\textsubscript{\alpha} solution at 270 nm, showed the fluorescence maximum at 319 nm (Fig. 7B), indicating the presence of tryptophan (19).

Copper-F\textsubscript{\alpha} complex failed to exhibit a characteristic absorption in the EPR spectrum even at a 0.3 mM concentration (based on copper analysis). Prior treatment of the sample with a few crystals of ferricyanide did not result in an EPR signal. A control with 1.0 mM cupric EDTA indicated that 0.05 mM cupric ion would have been detectable. The previous failure to observe an EPR spectrum of the copper-immunoglobulin complex in the presence or absence of ferricyanide (2) could have been explained in terms of spin-pairing of 2 cupric ions. However, the failure to observe a signal with the copper-F\textsubscript{\alpha}, which contains but a single copper atom, makes such an explanation less tenable.

The center wells contained antisera. In both experiments, Wells I and 4 contained the copper-IgG complex; Wells 2, 3, and 5 contained peptide Fractions I, II, and III, respectively. A, diffusion against antisera to the F\textsubscript{\alpha} fragment of normal human IgG. Fraction I formed a precipitin line of complete identity with the copper-IgG complex. Fractions II and III did not form precipitin bands. B, diffusion against antisera to the F\textsubscript{\beta} fragment of normal human IgG. Fraction II formed a precipitin band of complete identity with the copper-Sepharose complex. Fractions I and III did not form precipitin bands.

Fig. 5. UV and visible absorption spectra of the copper-F\textsubscript{\alpha} and apo-F\textsubscript{\alpha} fragments. The buffer was 0.01 M sodium phosphate, pH 7.2, containing 0.04 M sodium chloride. The temperature was 26°C and the volume 1.5 ml. UV spectra were obtained with a 0 to 2.0 slidewire and visible spectra with a 0 to 0.1 slidewire. A, copper-F\textsubscript{\alpha} fragment. A 24 $\mu$m protein solution was used for the UV spectrum and a 80.8 $\mu$m solution was used for the visible spectrum. B, apo-F\textsubscript{\alpha} fragment. A 11.6 $\mu$m protein solution was used for the UV spectrum and a 63 $\mu$m solution was used for the visible spectrum.
Copper-binding Site of a Myeloma Protein

Fig. 6. UV circular dichroism spectra of copper-Fab complex and apo-Fab. The buffer was 0.01 M sodium phosphate, pH 7.2, containing 0.04 M sodium chloride. The temperature was 25°C. Near UV spectra (200 to 250 nm) were obtained with a 2.0-mm pathlength cell and a sensitivity scale setting of 5 millidegrees/cm path length. (θ) = millidegrees/(cm·dm)·1. A, copper-Fab complex. A 93.4 μM protein solution was used for the near UV spectrum. B, apo-Fab. A 65 μM protein solution was used for the near UV spectrum. A 3.3 μM protein solution was used for the far UV spectrum.

Table I

<table>
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<tr>
<th>Amino acid composition of the copper-Fab fragment, S-carboxamidomethylated light chain, and S-carboxamidomethylated heavy chain of the copper-binding immunoglobulin</th>
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<td>Amino acid</td>
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α Determined as S-carboxymethylcysteine.
β Maximum value from 24- and 48-h hydrolyses. All other values are averages of 24- and 48-h hydrolyses, normalized to aspartic acid.
γ Extrapolated to zero time hydrolysis.
δ N.D., not determined.

The amino acid composition of acid-hydrayed copper-F_ab is presented in Table I.

Characterization of Apo-F_ab—Dialysis of copper-F_ab fragment against cyanide followed by gel filtration removed essentially all copper from the complex. The resulting apo-F_ab fragment is very similar to the copper-F_ab complex in terms of physical properties. The UV-visible spectrum (Fig. 5B) and fluorescence excitation (Fig. 7A) and emission spectra (Fig. 7B) of apo-F_ab are indistinguishable from the corresponding spectra of copper-F_ab. Moreover, apo-F_ab is very similar to copper-F_ab in terms of the large negative Cotton effect at 218 nm (which has been attributed in other proteins to the β structure) and the positive Cotton effect in the 277 to 292 nm region (Fig. 6). Thus, removal of the copper from the copper-F_ab complex appears to have little observable effect on structure.

Fig. 8 demonstrates that no immunological differences could be detected between apo-F_ab and copper-F_ab complex or between apo-IgG and copper-IgG complex. The apo-F_ab fragment (Wells 2 and 5) and apo-IgG (Well 4) form a precipitin line of complete identity with the copper-F_ab fragment (Well 1) and with the copper-immunoglobulin complex (Wells 3 and 6) when all are allowed to diffuse in agar gel against anticyper-F_ab antibodies (center well). This finding supports the conclusion drawn from the similarities of the physical properties of copper-F_ab and apo-F_ab that removal of copper results...
in no gross change in tertiary structure. According to this hypothesis, the conformation of the molecule is not dependent on the copper-protein bonds. The failure to observe immunological differences between the apoproteins and their copper complexes also suggests that the copper-binding site is not an antigenic determinant.

Reconstitution of Copper-Fab from Apo-F_{ab} and Cupric Ion—Copper-F_{ab} was reconstituted from apo-F_{ab} by the procedure used to reconstitute copper-immunoglobulin from apoinmunoglobulin (2). Apo-F_{ab} (1.33 mg) in a volume of 1.0 ml was applied to a column (1 x 54 cm) of Sephadex G-25 that had been equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 7.7 µM cupric nitrate. The column was eluted with the cupric nitrate containing Tris buffer. As expected for a successful Hummel and Dreyer run, the level of copper between the trough and the peak was equal to the level before the protein emerged from the column. The peptide peak was eluted with bound copper in a ratio of 1.02 atoms of copper/mol of F_{ab}. This ratio is consistent with the findings that the isolated and reconstituted copper-immunoglobulin complexes contain 1.8 atoms of copper/mol.

Preparation, Purification, and Characterization of Light and Heavy Chains—Treatment of copper-immunoglobulin complex with mercaptoethanol followed by reaction with iodoacetamide yielded the S-carboxamidomethyl derivatives of the light and heavy chain which were subsequently purified by gel filtration in aqueous propionic acid. This procedure has been employed with a number of IgG myeloma proteins for the preparation of derivatives of light and heavy chains in which the intrachain disulfide bonds are still intact (10). The isolated chains were shown to be homogeneous by disc gel electrophoresis. The molecular weights of the light and heavy chains were shown by SDS disc gel electrophoresis to be 28,000 and 56,000, respectively.

The amino acid compositions of the light and heavy chains are shown in Table I. Note that the light chain analyzed for only 1 S-carboxymethylcysteine residue, whereas the heavy chain analyzed for 3.7 S-carboxymethylcysteine residues (cysteines involved in intrachain disulfide bonds should not have been detected by this procedure).

Evidence for the Presence of a Sulfhydryl Group at the Copper-binding Site—Removal of copper from the copper-F_{ab} fragment resulted in exposure of one sulfhydryl group that was very reactive with 4,4'-dithiodipyridine. Reaction of thiols with this reagent gives the corresponding thiopyridone which has an absorption maximum at 324 nm (21). Fig. 9 shows that copper-F_{ab} reacted to only a small extent with dithiodipyridine, but that apo-F_{ab} reacted to a much larger extent and the absorption generated was proportional to the amount of apo-F_{ab} employed. From the slope of this curve for apo-F_{ab}, using values of 50,000 for the molecular weight of the apo-F_{ab} fragment and 1.4 x 10^{-6} for the molar extinction of the thiopyridone complex, the stoichiometry was calculated to be one reactive sulfhydryl group per apo-F_{ab} fragment. A single determination with reconstituted copper-F_{ab} (indicated by star) showed that the previously exposed sulfhydryl group was now largely protected as evidenced by the small extent to which the reconstituted complex reacted with the reagent.

After reaction with dithiodipyridine, apo-F_{ab} was unable to bind copper. No copper was bound to this modified apo-F_{ab} fragment when it was equilibrated with copper on a Sephadeox G-25 column under the exact conditions employed for the successful reconstitution of copper-F_{ab} from unaltered apo-
Prevention of copper binding by derivatization of the single sulfydryl group which is exposed by removal of the copper provides evidence that this sulfhydryl is indeed involved in the binding of copper. Alternatively, the inability of the derivatized apo-\(F_{ab}\) to bind copper may be a consequence of conformational changes resulting from derivatization, and the failure of copper-\(F_{ab}\) to react with dithioerythritol may be explained in terms of a conformational change resulting from binding of copper. However, any conformational changes which do occur as a consequence of copper binding are not detectable by the physical and immunological studies described above.

To ascertain whether the reactive sulfhydryl group detected in the apo-\(F_{ab}\) fragment was located on the light chain or on the \(F_{\lambda}\) fragment of the heavy chain, the thiol was labeled by reaction with \([^{14}C]\)iodoacetamide. Labeling of apo-\(F_{ab}\) was performed without prior cleavage of disulfide bonds so that only free sulfhydryl groups would be derivatized. Subsequent to the labeling reaction and the removal of labeling reagent, the \([^{14}C]\)-carboxamidomethyl-labeled apo-\(F_{ab}\) was reacted with mercaptoethanol and subjected to SDS-polyacrylamide disc gel electrophoresis. Essentially all of the radioactivity was detected in a polypeptide band with an \(R_f\) of 0.41. This polypeptide band migrated with the same \(R_f\) as homogeneous derivatized light chain isolated directly from the copper-immunoglobulin, suggesting that an \(-\text{SH}\) group of this chain is involved in the copper binding. The fact that, under these conditions, iodoacetamide reacted with apo-\(F_{ab}\) but did not react with the histidine residues of the light and heavy chains of the reduced copper-IgG complex (as evidenced by failure to detect carbamylhistidine upon amino acid analysis) provides further evidence that it is a sulfhydryl group which is being derivatized in this reaction.

The cysteine involved in copper binding does not appear to be derivatized upon subjecting the intact copper-immunoglobulin to iodoacetamide under conditions where only interchain disulfide bonds are reduced. The light chain derivative prepared in this way contains but a single \(S\)-carboxymethylcysteine (see Table I). Under these conditions, other IgG\(_1\) type immunoglobulins show one light chain carboxymethylcysteine (derived from the cysteine that was involved in disulfide linkage with the heavy chain). It is possible that in the case of the copper-immunoglobulin, the binding of copper to the thiol prevented the thiol from reacting with iodoacetamide and that the additional carboxymethylcysteine would be observed only upon amino acid analysis of carboxamidomethylated light chain derived from apo-IgG or apo-\(F_{ab}\). This is conceivable since the presence of copper prevents the \(-\text{SH}\) group of \(F_{ab}\), from reacting with dithioerythritol.

The derivative of the heavy chain obtained by reaction of the intact copper-immunoglobulin with iodoacetamide analyzed for 3.7 carboxymethylcysteine residues. Other IgG\(_1\) type immunoglobulins have analyzed for 3 such residues (derived from the 2 inter-heavy chain disulfide and the 1 disulfide linkage to the light chain). The detection of the additional 0.7 residue may reflect error in analysis, partial reaction of an \(-\text{SH}\) group on the \(F_{\lambda}\) fragment of the heavy chain.

**Nature of the Copper Complex**—The physical properties and the chemical studies give clues as to the nature of this very unusual copper complex. The absence of an EPR signal in the copper-immunoglobulin initially suggested that the molecule contained 2 spin-paired copper ions or copper ions. However, the absence of an EPR signal in the copper-\(F_{ab}\), and the reconstituted copper-\(F_{\lambda}\) complexes, which contain a single copper atom, makes unlikely the possibility of spin-pairing copper ions. Such a possibility would require that the \(F_{ab}\), fragment be capable of binding 2 copper atoms and that the isolated and reconstituted copper-\(F_{ab}\) complexes represent half-saturation of the complex. Although the present data cannot rule out such a possibility, we are working on the hypothesis that each of the two \(F_{ab}\), fragments of the immunoglobulin binds but a single copper atom.

The colorless nature of the immunoglobulin and \(F_{ab}\), copper complexes and the absence of strong absorption bands in their visible and near UV spectra are consistent with the presence of thiol-bound cuprous ions but not compatible with the presence of thiol-bound cupric ions. Proteins and small sulfhydryl compounds containing cupric ion bound to a sulfhydryl group have been shown to exhibit absorption bands in the 350 to 375 nm region (22). Cuprous sulfhydryl complexes, however, generally are either colorless or pale yellow and have no strong absorption band in the near UV region (23-26). The absence of an EPR signal after addition of ferricyanide to copper-immunoglobulin or copper-\(F_{ab}\) indicates that the copper is not oxidized by this agent to cupric ions. This observation would be compatible with the presence of nonoxidizable Cu(I) or Cu(III) (27, 28).

We do not understand the mechanism by which apo-\(F_{ab}\) binds cupric ions to give a copper complex which is EPR nondetectable. The reaction of cupric ion with mercaptides to give cuprous ion and disulfide is well documented (25, 29, 30). The cuprous form is stabilized by formation of a bond with sulfur.

\[
\text{Cu}^{2+} + 2RS^- \rightarrow \text{CuSR} + \frac{1}{2}(RS)_2
\]

In this reaction, reduction of the cupric to cuprous ion is accomplished with electrons from the mercaptide. In our study of the copper-\(F_{ab}\), fragment, only one sulfhydryl group was detected per \(F_{ab}\) fragment, and each \(F_{ab}\) fragment binds 1 copper atom. If copper is being reduced to the cuprous state, the question remains as to the source of electrons.

**Conclusions**—These studies demonstrate the binding of 1 atom of copper to each \(F_{ab}\), fragment. The inability to detect concanavalin A-binding carbohydrate residues in the copper-binding fragment suggests that the abnormal copper-binding affinity is a property of the unique primary structure of the \(F_{ab}\) fragment. Analysis of \(F_{ab}\), and apo-\(F_{ab}\), for \(-\text{SH}\) groups implicates an \(-\text{SH}\) group in the binding of copper. A labeling experiment indicates that this \(-\text{SH}\) group is located on the light chain. Other amino acid residues are undoubtedly also involved in the binding of the copper although no evidence for such interactions was obtained from physical studies. The oxidation state of the copper is left unanswered. The ability of apo-\(F_{ab}\), to react with a cupric ion to yield an EPR-nondetectable complex makes the copper-\(F_{ab}\), complex an excellent and most interesting model for studying EPR-nondetectable copper proteins.

**Acknowledgments**—We acknowledge the assistance of Dr. James A. Fee, Dr. Vincent Massey, Dr. Margaret Hunter, Dr. David Ballou, Mr. Larry Schopfer, and Dr. Richard Kirschner, all of The University of Michigan. We are most grateful to Ms. Linda Toler for her suggestions and editing.

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