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The Crystallizable Human Myeloma Protein Dob Has a Hinge-Region Deletion†

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ABSTRACT: During experiments to prepare heavy-metal derivatives of the crystallizable human IgG1 (κ) immunoglobulin Dob, it became apparent that this protein has several unusual features. (1) Instead of the four labile interchain disulfide bridges ordinarily found in IgG1, the Dob protein has only a single interchain disulfide bridge, which connects its two light chains. (2) The Dob heavy chain appears to be slightly smaller than a control γ1 chain, as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by gel filtration in guanidine. (3) The Dob heavy chain has three fewer residues of half-cystine than expected in γ1 chains. (4) The Dob IgG is relatively resistant to digestion with papain and trypsin; however, it is readily digested with pepsin, although at an unusual site. These findings suggest that some or all of the γ1 hinge region is missing in Dob. To localize the deletion, we prepared an $F(ab')_2$ fragment consisting of two heavy-chain pieces (Fd') noncovalently associated with the light-chain dimer. The Fd' piece was isolated and digested with trypsin. The sequence of the C-terminal tryptic peptide was Val-Ala-Pro-Glu-Leu-Leu-Gly-Gly-Pro-Ser-Val. Positions 2–11 of this peptide are identical with residue positions 231–240 of the γ1 chain. The N-terminal valine could be either Val-211 or Val-215 of the γ1 sequence. A tryptic peptide, Val-Asp-Lys-Lys, was also isolated from Dob Fd'; this sequence is not found in the variable region of the Dob heavy chain [Steiner, L. A., Garcia Pardo, A., & Margolies, M. N. (1979) *Biochemistry* (following paper in this issue)] but corresponds to positions 211–214 of the γ1 constant region. Therefore, the deletion cannot include these residues and must begin after Val-215; normal γ1 sequence resumes at Ala-231. The same 15-residue deletion has been found in two other IgG1 proteins, Mcg [Fett, J. W., Deutsch, H. F., & Smithies, O. (1973) *Immunochemistry 10*, 115] and Lec [Rivat, C., Schiff, C., Rivat, L., Ropartz, C., & Fougereau, M. (1976) *Eur. J. Immunol. 6*, 545]. Possible explanations for the occurrence of identical hinge-region deletions in three different immunoglobulins are suggested by recent experiments demonstrating that the three constant domains and the hinge region of mouse γ1 chains are each encoded by separate segments of DNA [Sakano, H., Rogers, J. H., Hüppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R., & Tonegawa, S. (1979) *Nature (London) 277*, 627].

Most information on the three-dimensional structure of immunoglobulins has been derived from X-ray diffraction studies of fragments obtained by proteolytic digestion [reviewed by Davies et al. (1975) and Poljak et al. (1976)]. However, to obtain information on the structural relations of the different immunoglobulin domains to each other and to gain insight into the functioning of the whole molecule, it is necessary to analyze the intact protein. Unfortunately, crystals suitable for X-ray studies have been obtained from only a few undigested immunoglobulins (Terry et al., 1968; Edmundson et al., 1970; Colman et al., 1976; Edmundson et al., 1978; Ely et al., 1978). The first of these to be investigated by crystallographic techniques was the human myeloma protein Dob, an IgG1 (κ) cryoglobulin (Terry et al., 1968). An electron density map at 6-Å resolution was consistent with a model in which the Fc fragment[1] forms the stem and the two Fab fragments form the arms of a T (Sarma et al., 1971). This interpretation was supported initially by electron micrographs of the protein crystals (Labaw & Davies, 1971) and, more recently, by a study in which domain coordinates derived from higher resolution studies of immunoglobulin fragments were fitted to the original diffraction data (Silverton et al., 1977).

Experiments were initiated in our laboratory to prepare heavy-metal derivatives of the Dob protein that might be useful for the X-ray diffraction studies. Our approach was based on earlier studies showing that divalent mercuric ions could be specifically inserted into the interchain disulfide bridges of immunoglobulins (Steiner & Blumberg, 1971). During the course of these experiments, it became clear that the covalent linkage between heavy and light chains was modified in the Dob protein. Instead of the four labile interchain disulfide

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[1] The nomenclature used for immunoglobulins is the following: γ1 is the heavy chain of the class IgG1; C_H1, C_H2, and C_H3 are domains or homology regions of the constant portions of the heavy chain; V_H is the variable domain of the heavy chain; the hinge region is the segment of heavy chain between the C_H1 and C_H2 domains; $F(ab')_2$ is a fragment, obtained by digestion with pepsin, that is composed of the two light chains and two fragments, called Fd', each comprising the N-terminal half of the heavy chain; Fab' is identical with $F(ab')_2$ but contains only one light chain and one Fd' fragment; Fab is a similar but slightly smaller fragment produced by digestion with papain; Fc is the fragment, obtained by digestion with pepsin, that is composed of the carboxy-terminal halves of the heavy chains; pFc' is a fragment, obtained by digestion with pepsin, that corresponds to the C-terminal half of Fc (the two C_H3 domains); details concerning the definition and preparation of these fragments are provided by Nisonoff et al. (1975).

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bridges expected in this subclass, there was only a single interchain bridge, which joined the two light chains. In the normal γ1 chain, the half-cystine residues that participate in interchain bonding all lie within a 10-residue segment of the "hinge region" between the C_H1 and C_H2 domains (Frangione & Milstein, 1967; Steiner & Porter, 1967; Gall et al., 1968). Accordingly, it was suggested that the anomalous interchain bonding in the Dob protein might be the consequence of a small deletion in this portion of the heavy chain (Lopes & Steiner, 1973). The evidence on which this suggestion was based is presented here. Further, we show that the Dob IgG has a number of unusual properties, all consistent with the postulated defect in the hinge region. Finally, we present evidence that 15 contiguous residues, normally found in the hinge region of γ1 chains, are absent in the Dob heavy chain. Exactly the same residues (positions 216–230)[2] are missing in two other IgG1 myeloma proteins, Mcg (Fett et al., 1973) and Lec (Rivat et al., 1976). No other structural anomalies in the Dob IgG have been found. In the accompanying paper, we show that the heavy chain has a complete variable region that is joined, without any missing segments, to the first constant domain (Steiner et al., 1979a).

Experimental Procedures

Materials. Plasma from patient Dob was supplied by Dr. W. D. Terry. Myeloma protein Gil [IgG1(λ)] and its Fc fragment were prepared as described (Steiner & Blumberg, 1971). Macroglobulin Ou [IgM (κ)] was a gift from Dr. H. Metzger. Enzymes were obtained from sources described by Steiner et al. (1979a). Iodoacetamide was recrystallized from water; [1-^{14}C]iodoacetamide (9.5 Ci/mol) was obtained from New England Nuclear. Urea was purified by stirring a 9 M solution with the mixed-bed resin Bio-Rad AG501-X8(D), 20–50 mesh, until the conductivity was <2 μmho. It was immediately adjusted to 1 M acetic acid and 8 M urea, stored at 4 °C, and usually used within 1 week. Guanidine hydrochloride was from Schwarz/Mann (Ultra Pure) or was purified from recrystallized guanidine carbonate according to Nozaki & Tanford (1967). Dithiothreitol and 5,5′-dithiobis(2-nitrobenzoic acid) were from Calbiochem, DEAE-cellulose (DE-52) was from Whatman, $NaDodSO_4$[3] was from Matheson Coleman and Bell, and Spectrapor No. 1 tubing (nominal molecular weight cutoff 6000–8000) was from Spectrum Medical Industries. The detergent *N,N*-dimethyllaurylamine oxide was from Onyx Chemical Co., Jersey City, NJ.

Purification of Dob IgG. Dob plasma (30 mL) was dialyzed overnight against 1 L of 0.15 M NaCl, 0.045 M $CaCl_2$, and 0.01 M Tris-HCl, pH 8.0. To prevent crystallization of the immunoglobulin, the dialysis was carried out at room temperature, and a few drops of toluene were added to the buffer. No clot formed and the buffer was changed to 1 L of 0.1 M Tris-HCl, pH 8.0. After about 3 h, a clot began to form, and the dialysis was continued against fresh 0.1 M Tris-HCl, pH 8.0, for another 20 h. The dialysis bag was warmed to 37 °C before removing the contents. The serum was separated from the clot by centrifugation and was passed through a 2.5 × 50 cm column of DE-52 equilibrated in 0.1 M Tris-HCl, pH 8.0. The effluent contained 2300 A_{278} units, i.e., 77 units/mL of plasma. Analysis of the purified protein by immunoelectrophoresis with an antiserum to human serum showed a single major precipitin band with cationic mobility (a "slow" IgG) with some diffuse anionic tailing. As will be demonstrated (see Results), electrophoresis in $NaDodSO_4$–polyacrylamide gels revealed that the preparation also contained a small amount of normal IgG, which could readily be removed by crystallization of the Dob protein. Crystallization was usually carried out at 4 °C in 0.1 M Tris-HCl, pH 8.0, at a protein concentration of ~20 mg/mL. The Dob protein has been reported to be IgG1 (κ) (Terry et al., 1968). Its Gm type is G1m (1, −17) and it is weakly positive for Km(3) (A. G. Steinberg, personal communication; Steiner et al., 1979b).

Preparation of Antisera. Antiserum to Dob IgG was obtained 5 months after immunization of a rabbit with 0.1 mg of purified protein and 12 days after a booster injection of the same amount of antigen. Antiserum that reacted with the Dob κ chain but not with the γ1 chain was obtained from a rabbit that had been immunized with 0.1 mg of macroglobulin Ou [IgM (κ)] 3 months previously and boosted 10 days before bleeding, again with 0.1 mg. Antigens were incorporated into complete Freund's adjuvant and were injected into the footpads. The preparation of antisera to Gil IgG, Gil Fab, Gil Fc, and normal human serum has been described (Steiner & Blumberg, 1971).

Reduction and Alkylation of IgG. Partial reduction (affecting mainly the labile interchain disulfide bridges) was carried out in 0.1–5 mM dithiothreitol, 0.2 M Tris-HCl ± 0.002 M EDTA, pH 8.0, followed by alkylation with recrystallized [1-^{14}C]iodoacetamide. Extensive reduction (to cleave all disulfide bridges) was carried out in 50 mM dithiothreitol, 7 M guanidine hydrochloride, pH 8.3, and 0.5 M Tris-HCl, followed by alkylation with nonradioactive iodoacetamide. The concentration of dithiothreitol was checked by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959). Details for these procedures are provided in the legends to Figures 1 and 3.

Digestion of IgG with Proteolytic Enzymes. The Dob and Gil proteins were treated with papain for 10 min to 4 h by using conditions previously shown to be effective with Gil IgG (Steiner & Blumberg, 1971). Both the native proteins and the partially reduced and alkylated proteins were incubated with trypsin (enzyme/substrate = 1% w/w) in 0.1 M Tris-HCl, pH 8.0, at 37 °C for 1–20 h. Digestion with pepsin (enzyme/substrate = 2–3% w/w) was in 0.05–0.1 M sodium acetate buffer, pH 4.5 or 4.25, at 37 °C. The results were evaluated by electrophoresis on cellulose acetate strips and in $NaDodSO_4$–polyacrylamide gels and by immunodiffusion.

Preparation of $F(ab')_2$ and Fd′. Dob and Gil IgG were digested with pepsin for 5–24 h. In each case, the solution became turbid as the digestion progressed but cleared when the digestion was terminated by adjusting the pH to ~8 with 2 M Tris base. The products of digestion were fractionated by gel filtration at 4 °C with Sephadex G-100 or G-150 in 0.2 M Tris–acetate, pH 8.2. As will be described under Results, the first peak eluted was $F(ab')_2$. To separate Dob Fd′ from the light-chain dimer, Dob $F(ab')_2$ was dialyzed into 1 M acetic acid and 8 M urea and subjected to gel filtration with Sephadex G-100 equilibrated at room temperature in the same solvent. Fractions were dialyzed individually with Spectrapor No. 1 tubing against 1 M and then 0.01 M acetic acid.

Reduction and Alkylation of Fd′. Fd′ (96 A_{278} units) in 0.01 M acetic acid was lyophilized and dissolved in 2.0 mL of 0.05 M dithiothreitol, 7 M guanidine hydrochloride, and 0.5 M Tris-acetate, pH 8.2. After 4 h at 37 °C, 45 μCi of

[2] Except where otherwise indicated, residues in the constant region of the Dob heavy chain are numbered according to the Eu γ1 sequence (Edelman, 1970).

[3] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; A_{278} unit, optical absorbance at 278 nm in a 1-cm path multiplied by the volume (in milliliters) of the solution.

[1-^{14}C]iodoacetamide in 0.1 mL of H_2O was added, and the solution was incubated at room temperature in the dark. After 15 min, 1.0 mL of 0.24 M nonradioactive iodoacetamide in the same buffer was added, and the incubation was continued for 30 min. [It has been reported that addition of alkylating agent in this sequence may enhance the labeling of protein thiol groups (O'Donnell et al., 1970).] An additional aliquot of dithiothreitol (50 μL of a 1 M solution) was then added to bind any unreacted iodoacetamide, and after another 30 min at room temperature, the solution was dialyzed with Spectrapor No. 1 tubing against 1 M and 1 mM acetic acid.

Determination of Protein Concentrations. These were based on absorbance at 278 nm. Extinction coefficients were obtained as follows. The purified Dob and Gil IgG were dialyzed into 0.05 M Tris-HCl, pH 8.0. The absorbance in the near-ultraviolet was measured, and the amino acid composition was determined from 24-, 48-, and 72-h hydrolysates. The following assumptions were made regarding the content of half-cystine and tryptophan. The light chains were assumed to contain 5 half-cystines, 3 tryptophans, and 214 residues in total. The half-cystine and tryptophan content of the Dob γ1 chain was taken from the sequence data established in this and the following paper (Steiner et al., 1979a) and from the known sequence of the constant region of the γ1 chain (Edelman, 1970). The Gil γ1 chain was assumed to contain 446 total residues, 11 half-cystines, and 7 tryptophans. The absorbances of 1 mg/mL solutions at 278 nm were as follows: Dob IgG, 1.66 ± 0.06; Gil IgG, 1.50 ± 0.06. [For Gil IgG an absorbance of 1.46 was reported previously, based on Kjeldahl N determinations (Steiner & Blumberg, 1971).] An extinction coefficient for extensively reduced and alkylated Dob Fd' was determined in a similar way except that the solvent was 1 M acetic acid. The absorbance of a 1 mg/mL solution was 2.2 ± 0.1. (The absorbance calculated from the sequence would be 2.11.) Extinction coefficients of 1.8 and 1.5 were assumed for the Dob and Gil Fab' fragments, respectively.

Digestion of Fd' with Trypsin and Purification of Peptides. To 85 A_{278} units of reduced, alkylated Fd' in 4.6 mL of 1 mM acetic acid, 1.1 mg of trypsin in 50 μL of 0.001 M HCl was added; the solution was warmed to 37 °C and adjusted to 0.1 M in NH_4HCO_3 by adding 0.23 mL of 2 M NH_4HCO_3. A precipitate formed immediately after the addition of the salt. The sample was incubated for 4 h at 37 °C with occasional agitation, 0.55 mg of trypsin was added, and the incubation was continued for another 2 h. Aliquots of the unfractionated digest were removed for digestion with carboxypeptidases A and B, and the sample was centrifuged. The precipitate was washed with 0.1 M NH_4HCO_3 and suspended in the original volume. Aliquots were removed from the supernatant and from the suspended precipitate for C-terminal analysis. The supernatant fraction, containing 12 A_{278} units and 46% of the total radioactivity, was lyophilized and taken up in 0.05 M acetic acid. A small amount of insoluble material was removed, and the remainder was fractionated by gel filtration as described in the legend to Figure 7. The eluted peptides were divided into two pools, as detailed under Results, and further purification was effected by paper chromatography and high-voltage electrophoresis; details for the latter procedures are provided in the following paper (Steiner et al., 1979a).

C-Terminal Analyses. Approximately 10 nmol of IgG, $F(ab')_2$, Fab', or Fd' was digested with 10 μg of carboxypeptidase A or carboxypeptidase B at 37 °C in 0.1–0.2 mL of 0.1 M NH_4HCO_3 for various periods of time. The digest was lyophilized, taken up in 1.1 mL of pH 2.2 citrate buffer, and applied directly to the 50-cm column of the Beckman amino acid analyzer. In later experiments with these substrates and also with fractions obtained after trypsin digestion of Fd', the amount of substrate was reduced to about 5 nmol, and amounts of enzyme between 0.5 and 5 μg were added in a total volume of 60 μL. After digestion for 1 h, samples were dried, taken up in 50–100 μL of pH 2.2 citrate buffer, and added to a Durrum D500 amino acid analyzer. The buffer for some digestions was made 0.1% in $NaDodSO_4$. Hydrazinolysis was carried out as described by Bradbury (1956). After extraction with benzaldehyde, the sample was rotary evaporated, taken up in pH 2.2 citrate buffer, and added to the 50-cm column of the Beckman analyzer.

Other Methods. N-Terminal and sequence analysis by the micro dansyl–Edman method and procedures for amino acid analysis are described in the following paper (Steiner et al., 1979a). Either a Beckman Model 120B or a Durrum D500 analyzer was used. The sequences of peptides T10[4] and T12, obtained by dansylation of residual peptide after each cycle of Edman degradation, were confirmed by amino acid analysis (subtractive method). Electrophoresis in polyacrylamide gels containing $NaDodSO_4$ was carried out as described by Steiner & Blumberg (1971). Samples that were analyzed without reduction were pretreated with 0.05 M iodoacetamide to prevent disulfide exchange in $NaDodSO_4$ (Virella & Parkhouse, 1973). Immunodiffusion was in 1% Ionagar No. 2S (Wilson Diagnostics, Glenwood, IL) on microscope slides with apparatus supplied by Gelman.

Results

Subunit Structure of Dob IgG. The susceptibility to reduction of the interchain disulfide bridges in Dob IgG was evaluated. The protein was incubated with various amounts of dithiothreitol and alkylated with recrystallized [1-^{14}C]-iodoacetamide. After dialysis, the extent of alkylation of each sample was determined from its absorbance at 278 nm and its radioactivity, together with the extinction coefficient of the IgG and the measured specific activity of the recrystallized [1-^{14}C]iodoacetamide. Another IgG1 myeloma protein (Gil), which had previously been studied in this laboratory, served as a control. Surprisingly, the results revealed a marked difference between the two proteins. As shown in Figure 1, in each case the extent of alkylation increased with concentration of reducing agent until a plateau was reached. However, there was a considerable difference in the rate of increase and, in particular, in the level of the plateau. For Gil IgG, the maximum level of alkylation was 7.4 mol of iodoacetamide per mol of protein, indicating that almost four disulfide bridges (presumably the two inter-heavy-chain and the two heavy–light chain bonds) had been cleaved. In contrast, for Dob IgG, the maximum alkylation was 2.1 mol of iodoacetamide per mol of protein, corresponding to the reduction of only a single disulfide bond. Exposure of either immunoglobulin to iodoacetamide without prior reduction resulted in very little uptake of radiolabel (less than 0.1 mol of iodoacetamide per mol of protein).

To obtain information about their subunit structure, we analyzed the various partially reduced samples by electrophoresis in $NaDodSO_4$–polyacrylamide gels. Unreduced Gil IgG migrated near the top of the gel; with increasing concentration of dithiothreitol, the protein was decomposed first into partial reduction products (various aggregates of heavy and light chains) and then mainly into free heavy and light

[4] Tryptic peptides are designated with the prefix T and are numbered in order, beginning from the N terminus of Fd'. The numbering is consistent with that used in the following paper (Steiner et al., 1979a).

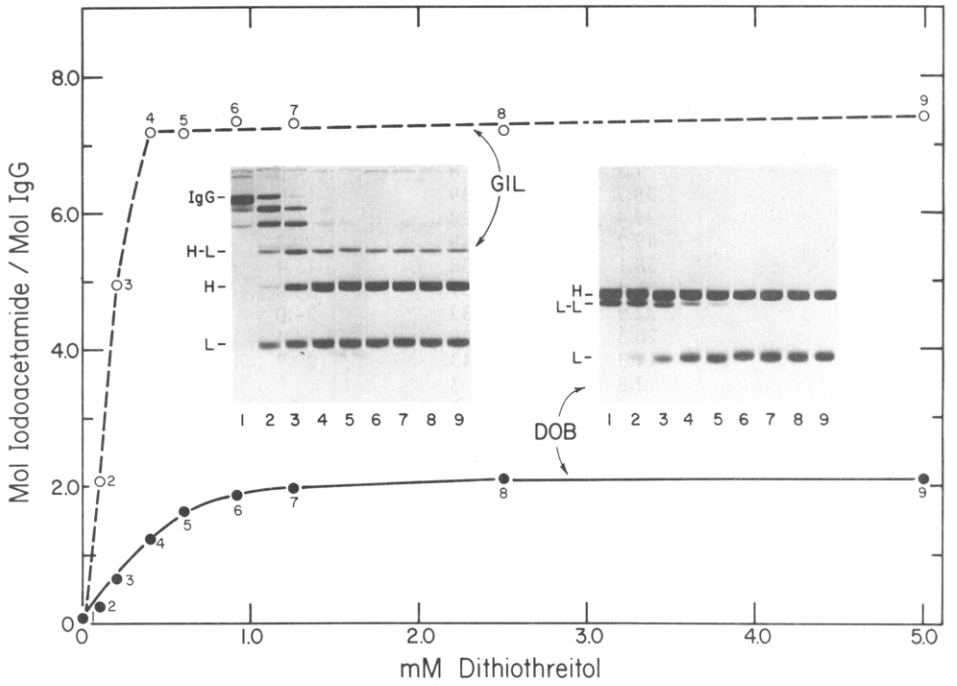

FIGURE 1: Titration of interchain disulfide bridges in Dob and Gil IgG. To 75 μL of IgG (A_{278} = 13.7) in 0.2 M Tris-HCl and 0.002 M EDTA, pH 8.0, various amounts of dithiothreitol were added to give the final concentrations indicated. After 1 h at 37 °C, 10 μL of recrystallized [1-^{14}C]iodoacetamide (358 cpm/nmol) was added; this was in 1.2 molar excess with respect to the concentration of sulfhydryl groups. After an additional hour at room temperature, 0.8 mL of 0.2 M Tris-HCl was added, and the samples were dialyzed against the same solvent until no radioactivity was detected in the dialysate; the extent of alkylation (expressed as moles of iodoacetamide per mole of IgG) was determined from their radioactivity and optical absorbance at 278 nm. The samples were also analyzed by electrophoresis in 6% polyacrylamide gels containing $NaDodSO_4$. Photographs of the stained gels are shown. H and L refer to heavy and light chains; H–L is a dimer of one heavy and one light chain and L–L is a dimer of light chains. The Dob IgG used in this experiment was recrystallized from protein that had been purified by DEAE-cellulose chromatography.

chains (Figure 1). In contrast, unreduced Dob IgG migrated as two closely spaced relatively fast-moving bands. The slower of these was in the approximate position of heavy chain, and its mobility was not affected by the reduction. However, with increasing reduction, the faster component gradually disappeared and was replaced by a component in the position of free light chain (Figure 1). Evidently, Dob IgG contains only a single disulfide bond that is labile to reduction under these conditions and that bond joins the two light chains. The heavy chains are not joined by covalent bonds to each other or to the light chains.

The effect of extensive reduction in $NaDodSO_4$ (to break intrachain as well as interchain disulfide bridges) on the mobility of the Dob and Gil heavy and light chains was also evaluated. As shown in Figure 2, there is a considerable difference in mobility between the partially and extensively reduced chains. Presumably, the cleavage of the intrachain disulfide bridges that occurs in the extensively reduced samples allows the polypeptide chain to unfold and assume a rodlike conformation (Reynolds & Tanford, 1970), thereby impeding its passage through the gel. The difference in mobility between the extensively reduced Dob and Gil heavy chains is consistent with a somewhat smaller (~2000) molecular weight for the Dob chain. After partial reduction, there was some difference in mobility between the Dob and Gil light chains, but this was no longer evident after complete reduction.

To provide additional evidence for the unusual interchain disulfide bonding in Dob IgG, the protein was partially reduced (in 5 mM dithiothreitol) and alkylated with [1-^{14}C]iodoacetamide, followed by extensive reduction in guanidine and alkylation with nonradioactive iodoacetamide. The heavy and light chains were then separated by gel filtration in 6 M guanidine hydrochloride. As a control, Gil IgG was subjected to exactly the same procedures. The heavy-chain peak, derived

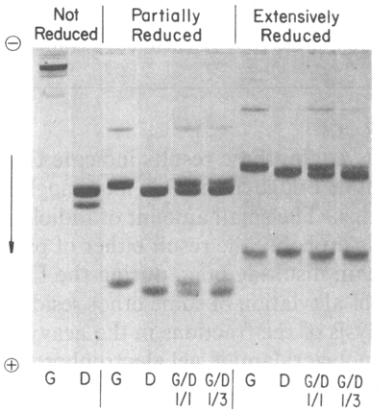

FIGURE 2: Electrophoresis of unreduced, partially reduced, and extensively reduced Dob and Gil IgG in 7.5% polyacrylamide–$NaDodSO_4$ gels. Partially reduced samples were from the titration experiment (Figure 1, no. 9, 5 mM dithiothreitol). Extensively reduced samples were treated with 50 mM dithiothreitol, 1% $NaDodSO_4$, and 50 mM Tris-HCl, pH 8.0, at 50 °C, for 1 h. The absorbance at 278 nm of each protein sample was 1.0; 16 μL of solution was taken for each gel except that the 1:1 mixtures (G/D, 1/1) were 8 μL of Gil plus 8 μL of Dob and the 1:3 mixtures (G/D, 1/3) were 4 μL of Gil plus 12 μL of Dob. The Dob IgG used in this experiment was recrystallized from protein that had been purified by DEAE-cellulose chromatography.

from the Dob IgG, contained 71% of the total A_{278} units eluted but only 22% of the total radioactivity (Figure 3). In contrast, the heavy-chain peak of Gil IgG contained 66% of the total absorbance units and 74% of the radioactivity. This distribution of radioactivity between Gil heavy and light chains is very close to that expected since, in the IgG1 subclass, three of the interchain disulfide bonds originate from each heavy chain and only one such bond originates from each light chain (Frangione & Milstein, 1967; Steiner & Porter, 1967; Gall

Table I: Amino Acid Composition of Dob Heavy Chain and Fd′

amino acid	no. of residues: heavy chain exptl[a]	heavy chain theoretical[b]	Fd′ exptl[c]	Fd′ theoretical[d]	Fc theoretical[e]
CmCys[f]	7.8	8	4.0	4	4
Asp	38.7	39	17.7	18	21
Thr	29.4	30	16.1	16	14
Ser	49.2	49	29.2	30	19
Glu	38.1	38	15.4	15	23
Pro	31.4	31	14.0	14	17
Gly	32.9	32	24.0	24	8
Ala	20.3	20	14.4	14	6
Val	44.3	43	20.9	21	22
Met	2.8	3	1.0	1	2
Ile	8.6	9	5.0	5	4
Leu	36.4	37	21.1	21	16
Tyr	17.9	18	8.9	9	9
Phe	14.6	15	8.1	8	7
His	9.8	10	4.1	4	6
Lys	28.6	29	11.1	11	18
Arg	12.0	12	6.1	6	6
Trp	nd[g]	11	nd	7	4
total		434		228	206
position in heavy chain		1-434		1-228	229-434

[a] Dob heavy chain (Figure 3, top, pool H) was hydrolyzed for 24, 48, and 72 h (four samples each). Each hydrolysate was analyzed in duplicate, and the values shown are the averages of the 24 runs, except that those for threonine and serine are logarithmic extrapolations to zero time, that for valine is the 72-h value, and that for isoleucine is the average of the 48- and 72-h values. The experimental values were normalized so that the sum of the residues less *S*-(carboxymethyl)cysteine and tryptophan is 415. [b] Values shown are sums of theoretical values for Fd′ and Fc segments (see footnotes *d* and *e*). [c] Fd′ (pool B, Figure 6) was extensively reduced and alkylated, and samples were hydrolyzed for 24 (3), 48 (2), and 72 h (2). Each hydrolysate was analyzed in duplicate, and the values shown are averages, except that those for threonine, serine, valine, and isoleucine were obtained as stated in footnote *a*. The experimental values were normalized so that the sum of the residues less *S*-(carboxymethyl)cysteine and tryptophan is 217. [d] Based on the following: residues 1-120, sequence of Dob heavy-chain variable region (Steiner et al., 1979a), residues 121-228, known sequence of Eu γ1 chain (Edelman, 1970) allowing for the 15-residue deletion in the Dob heavy chain and the Gm-related substitution of lysine for arginine at position 214 (Press & Hogg, 1970). Residues 121-228 in the Dob γ1 chain correspond to residues 118-215 plus 231-240 in the Eu γ1 chain. [e] Based on the Eu γ1 sequence with substitutions of aspartic acid for glutamic acid and leucine for methionine since Dob is G1m(1) and Eu is G1m(−1) (Rutishauser et al., 1970). Residues 229-434 in the Dob γ1 chain correspond to residues 241-446 in the Eu γ1 chain. [f] *S*-(Carboxymethyl)cysteine. [g] Not determined.

et al., 1968). Again, these results indicate that the only interchain disulfide bridge in the Dob immunoglobulin joins the two light chains. The small amount of radiolabel in the Dob heavy chain is probably the result either of partial reduction of an intrachain disulfide bond during the first stage of the reduction or of alkylation of some other residue in the heavy chain. (Analysis of the fractions in the heavy-chain peak by $NaDodSO_4$–polyacrylamide gel electrophoresis showed that they were not contaminated by light chains.) The Dob heavy chain was slightly retarded relative to the Gil heavy chain, also consistent with a slightly lower molecular weight for the Dob chain.

The foregoing results were all consistent with the hypothesis that a portion of the cystine-rich hinge region is missing in the Dob IgG. Consistent with this possibility was the finding that the Dob heavy chain contained only 7.8 residues of *S*-(carboxymethyl)cysteine (Table I), presumably originating from the reduction and alkylation of the four intrachain disulfide bridges. In contrast, the Gil heavy chain contained 11.1 residues of *S*-(carboxymethyl)cysteine, presumably derived from four intrachain and three interchain disulfide bridges. Moreover, the Dob heavy chain seemed somewhat deficient in proline [31.4 residues, compared to 35.6 for Gil and 37 reported for the Eu γ1 chain (Edelman, 1970)], a residue that is abundant in the hinge region.

Isolation and Characterization of $F(ab')_2$. To facilitate further structural studies of the Dob protein, we examined its susceptibility to proteolytic digestion. In comparison to Gil IgG, the Dob protein was relatively resistant to proteolysis with papain; both the native and partially reduced alkylated Dob immunoglobulin were also relatively resistant to trypsin. These findings were not surprising, as the preferred sites for cleavage by these enzymes are in the hinge region, the section of heavy chain apparently missing from the Dob molecule. However, it seemed possible that the position for peptic cleavage might be preserved in the Dob IgG since, as discussed below, mild proteolysis of IgG1 with pepsin is expected to degrade the heavy chain at a site several residues to the C-terminal side of the interchain disulfide bridges. Not only was it possible to fragment the Dob molecule with pepsin, but the digestion proceeded considerably more rapidly than the digestion of Gil IgG with pepsin at pH 4.5. After incubation with 2% (w/w) pepsin for 7 h at 37 °C, most of the Dob IgG was fragmented, whereas a considerable amount of Gil IgG remained undegraded.

The products obtained by pepsin digestion of Gil and Dob IgG were separated by gel filtration. When the 7-h digest of the Gil protein was fractionated on Sephadex G-150, three well-separated peaks were obtained, comprising 72, 8, and 20% of the eluted material. Since digestion was incomplete in 7 h, the Gil protein was also digested with pepsin for 24 h and applied to the same column; the first peak was now smaller (54%), the second was unchanged (8%), and the third was larger (38%). Analyses by $NaDodSO_4$–polyacrylamide gel electrophoresis and by immunodiffusion with antisera to Gil IgG, Fab, and Fc indicated that the first peak was a mixture of $F(ab')_2$ and undigested IgG. The second peak appeared to consist of pFc′, the fragment corresponding to the C_H3

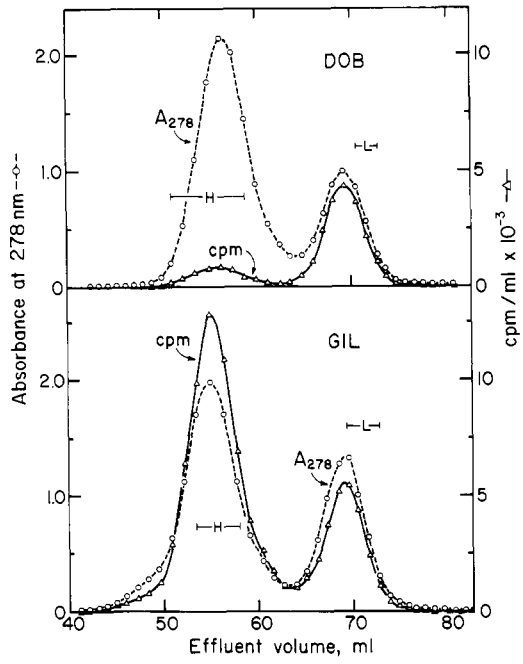

FIGURE 3: Separation of heavy and light chains from Dob and Gil IgG after partial reduction and alkylation with [1-^{14}C]iodoacetamide, followed by extensive reduction and alkylation with nonradioactive iodoacetamide. IgG (A_{278} = 19) in 1.8 mL of 0.2 M Tris-HCl, pH 8.0, was made 5 mM in dithiothreitol and incubated under N_2 for 1 h at 37 °C; a 1.3 molar excess (over sulfhydryl groups) of recrystallized [1-^{14}C]iodoacetamide (168 cpm/nmol) was added, followed by incubation for 1 h at room temperature in the dark. The samples were dialyzed against the same buffer until the dialysate contained no radioactivity and then into 7 M guanidine hydrochloride and 0.5 M Tris-HCl, pH 8.3. They were made 0.05 M in dithiothreitol, incubated for 4 h at 37 °C, and alkylated with 0.15 M iodoacetamide for 1 h at room temperature. Each sample was applied to a 1.5 × 90 cm column of Sepharose 6B equilibrated in 6 M guanidine hydrochloride. The volume of each fraction was determined from its weight, and the absorbance (278 nm) and radioactivity were measured; the fractions were also analyzed by polyacrylamide–NaDodSO$_4$ gel electrophoresis (not shown). Fractions containing heavy (H) or light (L) chains were pooled as indicated by the horizontal lines, dialyzed against 1 M acetic acid, and subjected to amino acid analysis.

domain. The third peak did not precipitate with any of the antisera and, presumably, contained only small peptides. When the first peak was digested with carboxypeptidase A, only leucine was released in significant quantity: 38 and 32% yield per 50 000 daltons for the material from the 7- and 24-h digests, respectively. This residue presumably originates from the pepsin-derived F(ab')$_2$ fragment, since intact IgG1 contains C-terminal glycine and serine (from the γ1 and λ chains), residues that are released poorly by carboxypeptidase A. (Hydrazinolysis of undigested Gil IgG confirmed that it contained C-terminal glycine.) These findings were consistent with previous work showing that digestion of IgG1 with pepsin at pH 2 produces a disulfide-linked core with Leu-234 at the C terminus of the heavy-chain fragment (Steiner & Porter, 1967). Givol & DeLorenzo (1968) found that a homologous leucine is the C-terminal residue in Fd' prepared from rabbit IgG.

When the 7-h pepsin digest of Dob IgG was subjected to gel filtration on the same column, a somewhat different elution profile was obtained. In addition to the three peaks found with the Gil digest, an extra peak was obtained, located between the first major peak and the peak presumably containing pFc'. Since the peaks were not completely resolved, another peptic digest of Dob IgG was prepared and fractionated by gel filtration with Sephadex G-100. The digestion with pepsin was carried out, in this case, at pH 4.25 and for 20 h. The elution profile of this digest is shown in Figure 4, together with NaDodSO$_4$–polyacrylamide gels showing the starting material (Dob IgG), the unfractionated pepsin digest, and the components in each of the first three peaks.

The IgG used in this experiment was purified by DEAE-cellulose chromatography, but it was not recrystallized. The two prominent bands in the NaDodSO$_4$ gel of undigested Dob IgG are the heavy chain and the light-chain dimer. The faint band near the top of the gel is probably normal IgG. The faint very fast-moving band is in the position of free light chain and is probably derived from molecules of Dob IgG in which the disulfide bond between the two light chains is not formed. Neither of these minor components is found in recrystallized Dob IgG (e.g., see Figures 1 and 2), and the mother liquor from the crystallization is enriched in both.

The identity of the components in the digest can best be understood by examining the products found in each peak. The last peak, D, had previously been shown not to contain any components detectable by staining the gels and is presumably composed of small peptides. Peak C contained a single fast-moving component, presumably pFc'. Peak B contained two closely spaced components, one in the position of free light chain and one just above; the latter was subsequently shown to be Fd', the heavy-chain portion of Fab'. The major components in the first peak, A, are Fd' and a group of closely spaced components migrating in the approximate position of heavy chain; the latter bands are the disulfide-bonded light-chain dimer, which tends to aggregate after isolation and usually appears as two or more bands. When the fractions in peak A were reduced and again analyzed by NaDodSO$_4$ gel electrophoresis, no component in the position of intact heavy chain was detected (insert, Figure 4). The minor, slowly migrating component in peak A is probably F(ab')$_2$ derived from the normal IgG.

Evidently, peak B contains the Dob equivalent of Fab' (Fd' plus light chain) and peak A contains the equivalent of F(ab')$_2$ (two Fd' fragments plus the light-chain dimer). In neither case are there covalent bonds between the Fd' fragment and the light chain, although noncovalent interactions hold the chains together. If the only difference between the protein fragments in peaks A and B is that the light chains in the former are joined by a disulfide bond, then this difference should disappear after reduction. Indeed, as shown in the insert to Figure 4, the patterns obtained after treatment with dithiothreitol in NaDodSO$_4$ were identical. It can be seen that the positions of Fd' and the light chain are reversed after reduction, Fd' migrating just *below* the light chain. (The evidence for this is not shown here but was provided by comparing these samples with Dob IgG that had also been reduced in NaDodSO$_4$.)

The presence of both F(ab')$_2$ and Fab' in the pepsin digest of the Dob IgG can be explained by our observation that the starting immunoglobulin preparation (purified by DEAE-cellulose chromatography but not crystallized) contained, in addition to covalently bonded light-chain dimer, a small amount of monomeric light chain. Since recrystallized Dob IgG does not contain free light chain, a pepsin digest prepared from this material should not contain Fab'. We have in fact shown by NaDodSO$_4$–polyacrylamide gel electrophoresis that a pepsin digest prepared from recrystallized Dob IgG does not contain a band in the position of free light chain.

Further evidence concerning the identity of the protein fragments in peaks A, B, and C was obtained by immunodiffusion with an antiserum to Dob IgG. As shown in Figure

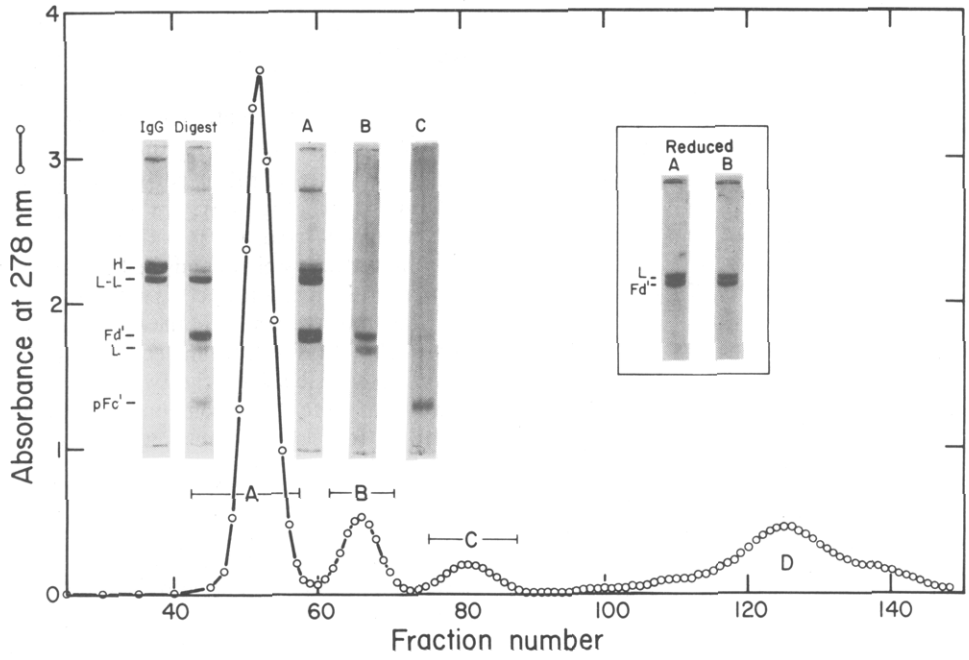

FIGURE 4: Gel filtration of peptic digest of Dob IgG and analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. DEAE-purified protein (740 A_{278} units) in 22 mL of 0.05 M sodium acetate buffer, pH 4.25, was incubated with 10 mg of pepsin for 20 h at 37 °C. After the pH was brought to ~8 with 2 M Tris, the digest was applied to a 5.0 × 140 cm column of Sephadex G-100 equilibrated at 4 °C in 0.2 M Tris–acetate, pH 8.2. The flow rate was 52 mL/h and fractions of 21.7 mL were collected. The IgG, the unfractionated peptic digest, and fractions from peak A (no. 52), peak B (no. 67), and peak C (no. 80) were analyzed without reduction by electrophoresis in 7.5% polyacrylamide–$NaDodSO_4$ gels. Photographs of the stained gels are shown. The insert shows the results of electrophoresis in 10% polyacrylamide–$NaDodSO_4$ gels of material in peaks A and B that had been reduced with dithiothreitol in the presence of $NaDodSO_4$. As described in the text, it was concluded that peaks A, B, and C are $F(ab')_2$, Fab′, and pFc′, respectively. Fractions from peaks A, B, and C were pooled as indicated by the horizontal lines.

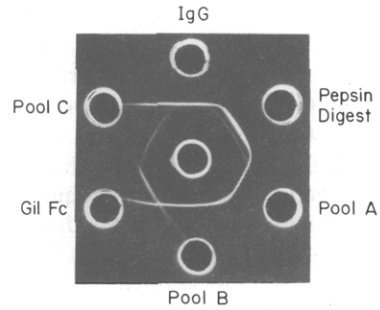

FIGURE 5: Antigenic analysis of fragments of Dob IgG produced by digestion with pepsin. Antigen concentrations were chosen to give sharp precipitin lines and ranged from 0.1 to 1 mg/mL. Pools A [$F(ab')_2$], B (Fab′), and C (pFc′) are those indicated in Figure 4. The center well contained antiserum to Dob IgG.

5, the material in pool A [$F(ab')_2$] was antigenically identical with the material in pool B (Fab′); the fragment in pool C (pFc′) was antigenically related, but deficient, to the papain Fc fragment obtained from Gil IgG and was unrelated to Fab′. The faint spur between Dob IgG and the pepsin digest indicates that antigenic determinants (presumably mainly in the C_H2 domain) have been degraded as a result of the digestion.

To determine the position of peptic cleavage, pool A [$F(ab')_2$] was digested with carboxypeptidase A. After 1 h of digestion, valine was obtained in approximately 85% yield (expressed as moles released per mole of Fab′); the yield of leucine was only 8%. Similar results were obtained with pool B (Fab′). Digestion of intact Dob IgG with carboxypeptidase A yielded small amounts of several residues, mainly glycine and serine, but very little valine. Hydrazinolysis of Dob IgG resulted in a quantitative release of glycine, presumably from the C-terminal position of the γ1 chain. When $F(ab')_2$ and Fab′ obtained from the 7-h pepsin digest of Dob IgG were digested with carboxypeptidase A for 2 h, a similar amount of valine was released but, in addition, phenylalanine was found in about 50% yield; when the time of digestion with carboxypeptidase A was reduced to 5 and 1 min, the yield of valine declined to 45 and 15%, respectively, but the yield of phenylalanine remained at about 50%. The results of the timed digestions suggest that the C-terminal sequence in this preparation of $F(ab')_2$ is –Val-Phe. The lower yield of phenylalanine than of valine after the 2-h digestion with carboxypeptidase A was puzzling but would be explained if $F(ab')_2$ has a "ragged" C terminus so that some molecules terminate with valine, whereas others terminate with the sequence Val-Phe. After 20 h of digestion with pepsin, only valine was found at the C terminus of both $F(ab')_2$ and Fab′, suggesting that pepsin gradually removed the terminal phenylalanyl residue. In an effort to determine the residue on the N-terminal side of the Val-Phe sequence, the Dob Fab′ fragment was subjected to hydrazinolysis *after* it had been digested to completion with carboxypeptidase A; no C-terminal residue was found in a significant amount.

The sequence Val-Phe occurs 3 times in the constant region of γ1 chains, at residue positions 125–126, 240–241, and 422–423 (Edelman, 1970). Only cleavage at the 240–241 site would be consistent with the data accumulated about $F(ab')_2$ and Fab′. Accordingly, it seemed that the site of cleavage of Dob IgG by pepsin was six to seven residues C-terminal to the cleavage of Gil IgG. In the γ1 chain there is no lysine or arginine between position 240 and the three cysteines (residues 220, 226, and 229) that normally form the interchain bridges and that are apparently deleted from the Dob γ1 chain. Therefore, the C-terminal tryptic peptide in the Dob Fd′ fragment should span the deletion.

The initial approach taken to isolate the C-terminal tryptic peptide from Dob Fd′ was to digest $F(ab')_2$ with trypsin, in an attempt to cleave a susceptible bond in the C-terminal region of Fd′ without degrading the entire protein. The

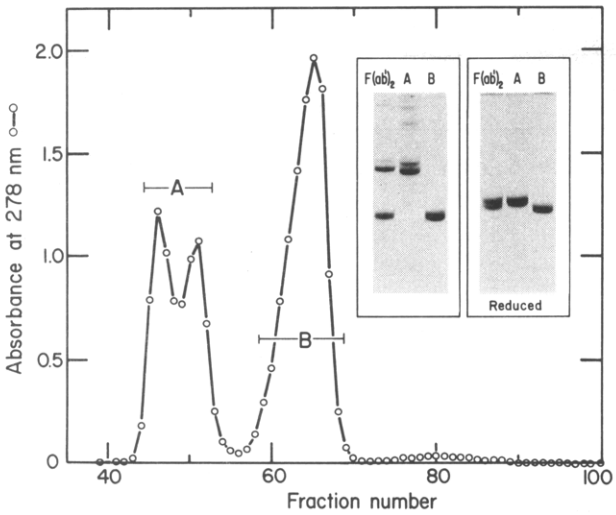

FIGURE 6: Separation of Fd′ from light-chain dimer by gel filtration. $F(ab')_2$ (380 A_{278} units) was dialyzed at 4 °C against 1 M acetic acid and 8 M urea and applied to a 5.0 × 145 cm column of Sephadex G-100 equilibrated at room temperature in the same solvent. The flow rate was 47 mL/h and fractions of 19.5 mL were collected. The starting material and representative fractions from peak A (no. 49) and from peak B (no. 63 or a pool), either unreduced (left box) or reduced with dithiothreitol in the presence of $NaDodSO_4$ (right box), were analyzed by electrophoresis in 10% polyacrylamide–$NaDodSO_4$ gels. The results indicate that peak A contains the disulfide-bonded light-chain dimer, which tends to form higher aggregates, and peak B contains the Fd′ piece. Fractions from peaks A and B were pooled as indicated by the horizontal lines.

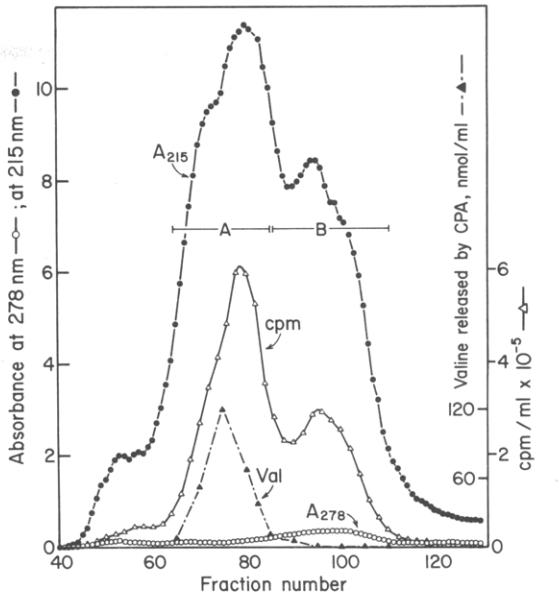

FIGURE 7: Gel filtration of the soluble tryptic peptides of Dob Fd′ on a 1.1 × 59 cm column of Sephadex G-25 in 0.05 M acetic acid. Fractions of 0.5 mL were collected, and the absorbance (278 and 215 nm) and radioactivity were determined. In addition, 50 μL of every fifth fraction was digested with 0.5 μg of carboxypeptidase A (CPA); the release of valine was used to localize the C-terminal peptide.

approach was applied first to Gil $F(ab')_2$; after mild reduction and alkylation of this fragment, digestion with trypsin did release the expected "hinge peptide" (residues 223–234). However, similar treatment of Dob $F(ab')_2$ did not lead to the cleavage of a C-terminal peptide. Reaction conditions of the trypsin digestion were varied by elevating the temperature (e.g., 60 °C for 20 min), but the C-terminal peptide was not released. $F(ab')_2$ and Fab′ were also digested with trypsin in the presence of 4 M urea, but no significant proteolysis occurred. Therefore, experiments were initiated to obtain the peptide from the isolated Fd′ piece.

Isolation of Fd′. To separate Fd′ from the disulfide-bonded light-chain dimer, $F(ab')_2$ from the 20-h pepsin digest was subjected to gel filtration with Sephadex G-100 in 8 M urea and 1 M acetic acid. As shown in Figure 6, a leading double peak (A) was well separated from a more retarded peak (B). Fractions across peak A were reduced in dithiothreitol and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis; all appeared to be identical and had the same mobility as the free light chain. When the fractions were not reduced, the more retarded ones (peak A, right) appeared to be composed primarily of light-chain dimers, whereas the less retarded fractions (peak A, left) contained mostly high molecular weight material (presumably aggregated light-chain dimers) that migrated near the tops of the gels. The fractions in peak B contained a single component, presumably Fd′. The results of $NaDodSO_4$ gel electrophoresis are also shown in Figure 6.

Digestion of fractions in peak A with carboxypeptidase A did not release valine or significant amounts of other residues; the amino acid composition of this material was indistinguishable from that of the free light chain that had been obtained by separating the Dob heavy and light chains (data not shown). Digestion of pool B with carboxypeptidase A released valine in about 60% yield; the amino acid composition of this material plus the theoretical composition of Fc corresponded closely to the composition of the isolated heavy chain (Table I). Fractions in peak A (after dialysis against the detergent, 1% *N*,*N*-dimethyllaurylamine oxide in 0.1 M Tris-HCl, pH 8.0) precipitated with an antiserum with specificity for human κ chains; fractions in peak B did not precipitate with this antiserum. These experiments provided an unambiguous identification of peak A with the light-chain dimer and peak B with Fd′. This result is, of course, consistent with the expected gel filtration behavior of these two components. The total absorbance at 278 nm of the material in peak B was significantly greater than that in peak A. This is consistent with the unusually high extinction coefficient determined for Dob Fd′ (see Experimental Procedures).

Localization of the Deletion. In attempts to release its C-terminal peptide, Fd′ was digested with trypsin under a variety of conditions, including solvents containing detergents or urea and elevated temperatures. However, no peptides were obtained. Accordingly, experiments were carried out to isolate the peptide from a complete tryptic digest of Fd′.

Fd′ was reduced in guanidine hydrochloride, alkylated with [1-^{14}C]iodoacetamide, and digested with trypsin. Most of the C-terminal peptide was shown, by digestion with carboxypeptidase A, to be in the soluble peptide fraction. The soluble peptides were separated by gel filtration with Sephadex G-25 into two pools, designated A and B (Figure 7). Pool A contained virtually all of the C-terminal peptide. Further purification of the peptides in both pools was effected by high-voltage electrophoresis and chromatography. A number of pure peptides were obtained; comparison with the known sequence of the γ1 constant region indicated which of these were derived from the constant region of the Dob heavy chain. The amino acid compositions and properties of these constant-region peptides are summarized in Table II.

Two of the peptides, T9 and T12, were critical in establishing the location and extent of the deletion. Peptide T12 was the only one containing C-terminal valine; it could, therefore, be placed at the C-terminal end of Fd′. Its sequence was Val-Ala-Pro-Glu-Leu-Leu-Gly-Gly-Pro-Ser-Val. The C-terminal 10 residues of this undecapeptide are identical with positions 231–240 in the γ1 chain (Figure 8). Residue 230

Table II: Amino Acid Composition of Soluble Tryptic Peptides from the Constant Region of Fd'

amino acid	no. of residues[a]					
	T7	T8	T9	T10	T11	T12[b]
CmCys[c]	–[d]	0.7 (1)	–	–	–	–
Asp	–	–	1.0 (1)	1.0 (1)	–	–
Thr	–	2.0 (2)	–	–	–	–
Ser	2.6 (3)	1.8 (2)	–	–	–	0.9 (1)
Glu	–	–	–	–	–	1.1 (1)
Pro	3.1 (3)	–	–	–	–	2.1 (2)
Gly	1.1 (1)	3.3 (3)	–	–	–	2.0 (2)
Ala	1.1 (1)	2.1 (2)	–	–	–	1.0 (1)
Val	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	–	2.1 (2)
Met	–	–	–	–	–	–
Ile	–	–	–	–	–	–
Leu	1.1 (1)	1.9 (2)	–	–	–	1.9 (2)
Tyr	–	–	–	–	–	–
Phe	1.0 (1)	–	–	–	–	–
His	–	–	–	–	–	–
Lys	0.9 (1)	0.9 (1)	2.0 (2)	1.0 (1)	1.0 (1)	–
Arg	–	–	0.1 (0)	–	–	–
total	(12)	(14)	(4)	(3)	(1)	(11)
position[e]	122–133	134–147	211–214	211–213	214	215, 231–240
Sephadex G-25 pool[f]	A	A	B	B	B	A
yield (%)[g]	23	38	27	48	33	30
mobility[h]	+0.28	+0.23	+0.52	N	+0.94	−0.28
N terminal	Gly	Ser	Val	Val		Val

[a] Experimental values were usually derived from one to two 24-h hydrolysates and are normalized to the total number of residues expected in the peptide. Values in parentheses are those expected from the known sequence of the γ1 chain (T7 and T8) and/or from the sequences determined in this paper (T9, T10, and T12). Where a blank appears, the expected value is zero. [b] Samples were hydrolyzed for 24 and 72 h. Values are averages except for serine which is the 24-h value and leucine and valine which are 72-h values. [c] *S*-(Carboxymethyl)-cysteine; determined from the specific activity of hydrolyzed peptide. [d] (–) represents <0.1 residue. [e] Position in the Eu γ1 chain (Edelman, 1970). [f] See Figure 7. [g] Expressed relative to the amount of Fd' digested with trypsin. [h] Mobility in high-voltage electrophoresis at pH 6.5, expressed relative to aspartic acid. Positively charged peptides are designated (+); negatively charged peptides are designated (–); N denotes neutral.

in the γ1 sequence is proline, but in peptide T12 the residue N-terminal to Ala-231 is valine. In the γ1 sequence the first valine N-terminal to residue 231 is at position 215 and the next is at position 211. Either of these valyl residues could be at the N terminus of a tryptic peptide since position 210 in the γ1 chain is lysine and position 214 is either lysine or arginine (according to the allotype). The sequence of peptide T9 was Val-Asp-Lys-Lys. Such a sequence occurs in the γ1 constant region only at residue positions 211–214 (Edelman, 1970) and does not occur in the Dob V_H region (Steiner et al., 1979a). Therefore, the deletion cannot include these positions. (Similarly, the related peptide, T10, with sequence Val-Asp-Lys can be placed at positions 211–213.) Accordingly, it was concluded that the N-terminal valine in peptide T12 must correspond to position 215 of the γ1 chain and that the 15 residues between Val-215 and Ala-231 (i.e., positions 216–230) are missing in Dob. This 15-residue deletion is consistent with the amino acid composition of Fd', as shown in Table I. The position and sequence of the peptide used to establish the location of the deletion, as well as other structural features of the Dob protein, are shown in Figure 8.

Screening Procedure for Other IgG1 Myeloma Proteins with Hinge-Region Deletions. In addition to Dob, two other IgG1 myeloma proteins have been found to have exactly the same deletion, residues 216–230 of the hinge region (Fett et al., 1973; Rivat et al., 1976). To identify other such proteins, it would be useful to have a simple method for detecting them in myeloma sera. In denaturing solvents, an IgG1 protein with a hinge-region deletion dissociates into heavy chains and light-chain dimers. As shown in Figure 9, this anomaly is readily detected when whole myeloma serum is examined by electrophoresis in $NaDodSO_4$–polyacrylamide gels since, after suitable dilution, the only prominent bands are those corresponding to the myeloma protein and to serum albumin. In

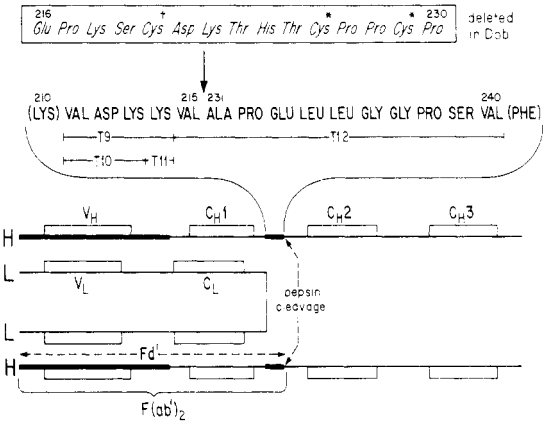

FIGURE 8: Diagrammatic representation of the structure of Dob IgG. The heavy (H) chains are monomeric and the light (L) chains form a dimer linked by a disulfide bridge. The disulfide-bonded loops in each domain are indicated. The properties of tryptic peptides T9–T12 are described in the text and are summarized in Table II. The thickened lines denote those sections of the heavy chain whose sequence has been determined in this and the following paper (Steiner et al., 1979a). The sequence of the heavy-chain segment between C_H1 and C_H2 is shown, indicating the location of the hinge-region deletion. The residues that are missing from the Dob chain (216–230) are indicated in the box; Cys† is the residue that ordinarily forms a disulfide bond to the light chain; Cys* are the residues that ordinarily form disulfide bonds with the other heavy chain. The numbering used here is that of the Eu heavy chain (Edelman, 1970).

the other human IgG subclasses, the disulfide bridges between the heavy chains also originate in the hinge region, but the bridge between the heavy and light chains does not [see Nisonoff et al. (1975) for references]. A hinge deletion in these proteins would probably lead to dissociation, in appropriate solvents, into half-molecules (heavy–light-chain pairs); these could also be detected by $NaDodSO_4$–polyacrylamide elec-

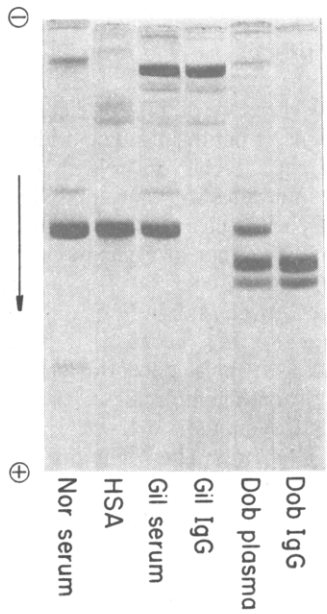

FIGURE 9: Screening human sera for myeloma proteins with the hinge-region deletion. Serum (or plasma) and proteins were analyzed, without reduction, by electrophoresis in 6% polyacrylamide–$NaDodSO_4$ gels. Sera were diluted 1:100; the proteins were approximately 0.7 mg/mL; 10 μL was applied to each gel. Nor serum is normal human serum, and HSA is human serum albumin. The difference between a typical IgG myeloma protein (Gil) and the atyptical Dob IgG is clear, even when unfractionated serum or plasma containing these proteins is tested.

trophoresis.

Discussion

Evidence for the Hinge-Region Deletion. The unusual structure of the crystallizable immunoglobulin Dob became apparent in experiments designed to substitute divalent mercuric ions into its interchain disulfide bridges. Immunoglobulins of the IgG1 subclass ordinarily have four such bridges, two joining the pair of heavy chains and one between each heavy–light-chain pair. The Dob molecule, however, has only a single interchain bond, which connects the two light chains (Figures 1–3 and 8). Further, the behavior of the extensively reduced Dob heavy chain in $NaDodSO_4$–polyacrylamide gel electrophoresis and in gel filtration in guanidine indicated that its molecular weight is slightly less than that expected for a γ1 chain (Figures 2 and 3). Amino acid analysis of the Dob heavy chain showed that it contains three fewer residues of half-cystine than expected in γ1 chains (Table I). The Dob protein was also unusual in its susceptibility to proteolytic cleavage, as discussed below. These findings are consistent with a small deletion in the Dob heavy chain, eliminating some or all of the hinge region, including the three half-cystines that normally participate in interchain bonding. Unable to bridge to the heavy chain, most of the Dob light chains form dimers connected by a single disulfide bond. Although the heavy chains are not covalently bonded to the light chains, the Dob molecule was reported to sediment at 7.37 S (Terry et al., 1968), indicating that it is associated as a tetramer in nondissociating solvents. Direct evidence for a deletion of 15 residues (positions 216–230) in the Dob hinge region was obtained by isolating the C-terminal tryptic peptide of Fd′ and showing that its N-terminal residue corresponds to Val-215 and the remaining 10 residues correspond to positions 231–240 of the γ1 chain. In analyzing the structure of the constant portion of the Dob heavy chain, we have made the assumption that it is a γ1 variant and not a new subclass that happens to share allotypic determinants or other structural features with the γ1 chain (see also Genetic Basis for Immunoglobulin Variants).

Susceptibility to Proteolytic Digestion. The Dob IgG was relatively resistant to digestion with papain and trypsin. This observation is consistent with a hinge deletion since the primary sites for cleavage by these enzymes are located between the interchain bond joining the heavy and light chains and the two bonds that link the heavy chains (Frangione & Milstein, 1967; Steiner & Porter, 1967; Gall et al., 1968; Wang & Wang, 1977). Glueck et al. (1972) reported that the Dob protein was split into Fab and Fc fragments by papain; the fragments were not characterized in detail, and it is possible that the enzymatic degradation occurred at secondary sites not included in the deletion [see Wang & Wang (1977) and Utsumi (1969)]. The Dob IgG was readily split by pepsin at pH 4.25–4.50; indeed, $F(ab')_2$ was released more rapidly from the Dob than from the Gil protein. The susceptibility of Dob IgG to pepsin is not suprising, since the expected site for peptic cleavage of IgG1 (Leu-234, five residues C-terminal to the second inter-heavy-chain bridge) is not included in the deletion. Analysis of the products of pepsin cleavage indicated, however, that cleavage had occurred not at Leu-234 but at Val-240 and Phe-241. The unusual location and the enhanced rate of peptic cleavage in Dob may be due to an altered conformation secondary to the deletion, resulting in the exposure of a new and favorable site for degradation by the enzyme.

It is interesting that cleavage of the Dob heavy chain by pepsin occurred in a sequence, Pro-Ser-Val-Phe (positions 238–241), that is found with little or no variation near the start of the constant regions of virtually all human, mouse, and rabbit light chains, as well as near the beginning of each constant domain of most heavy chains in these species and in guinea pigs (Dayhoff, 1976; Putnam, 1977). In some proteins, as in Dob, this sequence seems to be a preferential site for digestion by pepsin. Thus, peptic cleavage of a human κ Bence-Jones protein into variable and constant domains occurred at the homologous phenylalanyl residue (position 116) when the digestion was carried out at 37 °C to produce an intact variable-region fragment or when it was carried out at 55 °C to produce an intact constant-region fragment (Seon et al., 1973). Digestion of an intact IgG1 (κ) myeloma protein with pepsin in 8 M urea, pH 3.6, split the κ chain at the same position (Parr et al., 1976). Cleavage at this site in the first constant domain of both heavy and light chains also occurred when IgM was digested with pepsin at 4 °C (Lin & Putnam, 1978). A consequence of the shortened or missing hinge region in the Dob IgG may be that the conformation at the beginning of the C_H2 domain now resembles that ordinarily found at the beginning of C_H1, thereby shifting the position of the peptic split from Leu-234 to Val-240 and Phe-241 in the conserved sequence at the beginning of C_H2. Another factor in determining the position of enzymatic cleavage could be the location of carbohydrate chains; e.g., in X-ray diffraction studies of an isolated Fc fragment, carbohydrate was found to cover certain residues in this region, including Phe-241 (Deisenhofer et al., 1976b). The carbohydrate in Dob, which also occurs in this region of the molecule (Silverton et al., 1977), may occupy a slightly different position.

Expression of Allotypic Determinants. Another indication of a change in conformation resulting from the deletion in the hinge region was obtained by analysis of the allotypic determinants of the Dob protein and serum, carried out by Dr. A. G. Steinberg. The Dob immunoglobulin was obtained from a Negroid patient; accordingly, the allotypic determinants G1m(1) and G1m(17) would be expected in molecules of the IgG1 subclass (Natvig et al., 1967). Indeed, serum from the

Dob patient was positive for both of these markers (A. G. Steinberg, personal communication). However, the isolated myeloma protein was G1m (1, -17) (A. G. Steinberg, personal communication; Steiner et al., 1979b). Since the lysyl residue at heavy-chain position 214, which is correlated with G1m(17), is present in Dob, the deficiency in antigenic expression is probably related to an altered conformation in this region of the molecule consequent to the hinge deletion. The determinant itself might be modified or it could be masked, e.g., because of closer contact between the C_H1 and C_H2 domains or by the dimerized light chains. It had previously been shown that the Gm marker associated with heavy-chain position 214 requires the presence of the light chain for its expression (Polmar & Steinberg, 1964). The light-chain antigenic marker, Km(3), was detected in the isolated Dob protein, although its expression was somewhat weaker than usual (Steiner et al., 1979b).

Three-Dimensional Structure. The most direct approach to evaluate the effects of the hinge deletion on conformation is to analyze crystals of both normal and deletion proteins by X-ray diffraction. The first apparently normal (i.e., with an intact hinge) immunoglobulin to be studied by this method was the IgG1 myeloma protein Kol (Colman et al., 1976). Unexpectedly, no interpretable electron density was found C-terminal to the inter-heavy-chain disulfide bridges, presumably because the Fc part of the molecule was disordered in the crystal lattice. It was suggested (Colman et al., 1976; Huber et al., 1976) that this disorder is a consequence of inherent flexibility in the immunoglobulin molecule, the Fc region being able to assume a number of positions with respect to the Fab arms. Nonetheless, the possibility remained that the Fc region influences the structure of the Fab part of the molecule. Ely et al. (1978) compared the IgG2 myeloma protein Zie, which also has an intact hinge, with the divalent $F(ab')_2$ fragment derived from this protein. Crystals of the parent protein and of the fragment were nearly isomorphous, and their diffraction patterns were very similar. It was therefore concluded that the Fc region does not contribute significantly to the diffraction pattern of the whole Zie protein; moreover, the Fab regions have similar conformations in the fragment and in the parent molecule. The structure of the Fab region of protein Kol is also very similar in the intact protein and in the isolated fragment (Matsushima et al., 1978).

The original crystallographic studies of the Dob IgG at 6-Å resolution (Sarma et al., 1971) showed the overall molecular boundary and led to the suggestion that the molecule is T-shaped, with the Fc fragment forming the stem and the two Fab fragments forming the arms of the T. This overall configuration has been confirmed in a recent reexamination of the structure using known domain coordinates from immunoglobulin fragment structures (Silverton et al., 1977). The most striking difference between the results obtained with Dob and those with the Kol and Zie proteins is that electron density corresponding to Fc was clearly evident in the Dob protein. Indeed, the quaternary structure of the Dob Fc region, except for small differences in chain symmetry, is identical with that of an isolated Fc fragment studied by Deisenhofer et al. (1976a,b). Low-resolution electron density maps of another IgG1 protein with a missing hinge region (Mcg) also showed an ordered Fc region (Edmundson et al., 1978). Perhaps, the mobility of the Fc portion of the molecule, relative to the Fab arms, is reduced as a result of the hinge deletion. In contrast to the Dob protein, the Mcg molecule appears to be Y-shaped with the two Fab arms fairly close together, implying that even hingeless IgG proteins can assume different conformations (Edmundson et al., 1978).

Crystallizability of the Dob Protein. Dob IgG, purified from serum by chromatography on DEAE-cellulose, appeared to contain two varieties of hinge-deleted molecules; these differed in whether or not the disulfide bridge between the light chains had formed. However, crystals of the Dob protein contained only molecules with covalently bonded light chains (compare $NaDodSO_4$ electrophoretograms of Dob IgG in Figures 1 and 2 with that in Figure 4). Evidently, the two varieties of IgG did not cocrystallize; molecules without the disulfide bridge between the light chains did not form independent crystals, either because of inherent molecular properties or because their concentration was too low. After digestion with pepsin, the molecules with dimeric light chains formed $F(ab')_2$ fragments and those with monomeric light chains formed Fab' fragments. The ratio of $F(ab')_2$ to Fab' can be determined by gel filtration (e.g., Figure 4), and reflects the distribution of the two types of molecules in the IgG preparation. Why the disulfide bridge formed in most but not all of the molecules is not clear. Free light chains did not appear in samples of recrystallized protein that were stored for 20 months at -18 °C.

The incomplete formation of disulfide bonds between two presumably identical light chains is reminiscent of the pattern of disulfide-bond formation in Bence-Jones proteins; these light chains, excreted in the urine of patients with multiple myeloma, may be a mixture of disulfide-bonded dimers and monomers (Bernier & Putnam, 1963; Gally & Edelman, 1964). In the dimeric form, the half-cystine residue that, ordinarily, is involved in the interchain link to the heavy chain forms instead a disulfide bridge with the corresponding residue in the second light chain; in the monomeric form, the sulfhydryl group of this residue may be blocked with cysteine (Milstein, 1964). We have not determined whether the terminal half-cystine residue in the monomeric Dob κ chain has a free sulfhydryl group or whether it is blocked.

The power of crystallization to discriminate between closely related molecules was also demonstrated in the studies of the $F(ab')_2$ fragment of the IgG2 (κ) protein Zie (Ely et al., 1978). Two Fd' fragments, a major component and a slightly larger minor component, were obtained after digestion with pepsin. Crystals of $F(ab')_2$ contained light chain and the smaller Fd' fragment, whereas the mother liquor contained both Fd' components.

Biological Activity of the Dob Protein. The hinge deletion makes the Dob protein a good candidate for studies on the importance of this region in the biological functioning of immunoglobulins. Feinstein & Rowe (1965) originally suggested that complement fixation may require movement of the antibody molecule about a "hinge point". This was consistent with the observation of Schur & Christian (1964), confirmed by Press (1975), that mild reduction of rabbit antibody diminishes its complement-fixing capacity. Probably, the critical structural change is reduction of the disulfide bridge that joins the hinge regions of the two heavy chains. The studies of Isenman et al. (1975) with human immunoglobulins also point to a role for the hinge-region disulfides in complement binding; reduction of the inter-heavy-chain bonds of IgG1 destroyed reactivity with C1, the first component of the classical complement path. Interestingly, reduction of these same bonds did not affect the C1 binding of the *isolated* Fc fragment. It was also found that the Fc fragment of human IgG4 can bind C1, although the intact IgG4 cannot. Isenman et al. (1975) suggested that the Fab fragment in some immunoglobulins may sterically block the C1 binding site.

Whether or not this occurs may be related to the sequence and disulfide bonding and hence the conformation of the hinge region. The activity of the Dob protein (either aggregated or not) in a standard complement fixation test or in binding to C1 could be determined (Augener et al., 1971; Isenman et al., 1975).

Another biological function of immunoglobulins that might be affected by the hinge deletion is binding to cells, presumably mediated by surface receptors for the Fc portion of the molecule. Ciccimarro et al. (1975) reported that the Dob protein inhibited rosette formation between human monocytes and human erythrocytes coated with Rh antibodies. In contrast, it has recently been found that the Dob IgG did not inhibit the binding of radiolabeled trimers of rabbit IgG to the macrophage-like murine tumor, $P388D_1$, although the Gil IgG myeloma protein did inhibit this binding (J. A. Titus and D. M. Segal, personal communication). Additional experiments, testing the binding of the Dob protein to a variety of cell types, are required to clarify its cell-binding functions.

Hinge Deletion Proteins. In addition to the Dob protein, two other IgG1 molecules, Mcg (Deutsch & Suzuki, 1971; Fett et al., 1973) and Lec (Rivat et al., 1976), have exactly the same deletion, residues 216–230 of the heavy chain. As might be expected, several of the unusual properties of the Dob IgG are shared by these proteins. The Mcg and Lec proteins also have free heavy chains noncovalently associated with disulfide-bonded light-chain dimers and do not express the Gm antigenic determinant associated with residue 214. The Lec IgG did not express the Km (formerly Inv) determinant that would normally be associated with its light chain. This deficiency in light-chain antigenicity did not appear to result from any defect in the primary structure of the light chain and, presumably, reflects a change in conformation secondary to the hinge deletion, perhaps a consequence of light-chain dimerization. The Km(3) allotype was detected in the Dob IgG, but its expression was weak. The Mcg protein also crystallizes (Edmundson et al., 1970), but no comment was made by Rivat et al. concerning the crystallizability of the Lec protein. No other abnormalities in the primary structure of these proteins have been reported; the Dob heavy chain has an intact variable region that is joined in the usual way to the C_H1 domain (Steiner et al., 1979a).

The identity of the deletions in these proteins raises the question of the exact extent and location of the "hinge region". This term has come to be used to designate the region of the heavy chain between Fab and Fc, which is rich in proline, contains some or all of the interchain disulfide bridges, is particularly susceptible to enzymatic cleavage, and may impart flexibility to the IgG molecule [see discussions by Cathou & Dorrington (1975) and Huber et al. (1976)]. The hinge can also be identified by its lack of homology with the immunoglobulin domains. In one alignment of the domains of the $\gamma1$ chain, the extra segment between the C_H1 and C_H2 domains comprises residues 221–233 (Edelman, 1970). These positions overlap but are slightly C-terminal to the deleted section (residues 216–230) of the Dob, Mcg, and Lec $\gamma1$ chains. Another definition of the hinge region may emerge from studies of the organization of the DNA coding for the heavy chain (see Genetic Basis for Immunoglobulin Variants).

Other Immunoglobulin Variants. Immunoglobulins with internal deletions in both heavy and light chains have been described [reviewed by Franklin & Frangione (1975)]. An interesting feature of a number of these variants is that the deletions begin or end at or near a domain boundary. Thus, some human proteins have deletions encompassing parts of the V_H region and all of the C_H1 domain (Franklin & Frangione, 1975; Franklin et al., 1979). A deletion involving the C_H1 domain of a mouse IgG1 myeloma protein (MOPC 21, variant IF2) has also been described (Milstein et al., 1974; Adetugbo, 1978b). In this group of variants, normal sequence usually resumes just before the hinge region at residue Glu-216 of human $\gamma1$ chains or at a homologous position in other classes or species [e.g., Val-215 in mouse $\gamma1$ chains (Milstein et al., 1974; Adetugbo, 1978b)]. This is the same residue that marks the beginning of the missing segment in the three hinge deletion proteins.

The human λ Bence-Jones protein Sm has a deletion of the C-terminal portion of the variable region, with normal sequence resuming at or near the junction between the variable and constant domains (Garver et al., 1975). The light chain of the IgG1 (κ) myeloma protein Sac has a 68-residue deletion that is confined to the variable region (Parr et al., 1972); although its exact location is not completely clear, Franklin et al. (1979) have suggested that this deletion terminates at a residue that corresponds to a site of somatic DNA recombination (see the discussion under Genetic Basis for Immunoglobulin Variants). A fragment consisting of the constant region of a mouse light (κ) chain (Kuehl & Scharff, 1974) may also be a deletion variant since it is synthesized as a precursor with a κ leader peptide (Rose et al., 1977; Burstein & Schechter, 1978).

Genetic Basis for Immunoglobulin Variants. An initial question is whether these unusual proteins result from defects in the structure, transcription, or translation of genes coding for immunoglobulin chains or whether they represent normal, but infrequently expressed immunoglobulin classes. In support of the latter possibility, Lam & Stevenson (1973) reported that some molecules with the properties of heavy-chain disease proteins could be isolated from normal human serum. Nevertheless, its seems unlikely that all of these proteins should be poorly expressed normal immunoglobulins since there are many different variants, and it is not apparent why they should all have been preserved during evolution only to be expressed at such a low level. The possibility that the three known hinge deletion proteins represent a minor IgG subclass is perhaps somewhat more likely since the constant regions of these three γ chains seem to be identical (although they have not been completely sequenced), and their level of expression may be comparable to that of the ϵ chain.

Most investigators have considered that these variants are the results of abnormalities in the structure or expression of immunoglobulin genes. A number of hypotheses have been advanced to explain the origin of these proteins and, in particular, to account for the observation that the deletions often begin or end at domain boundaries, e.g., residue 216 at the beginning of the hinge region (Fett et al., 1973; Milstein et al., 1974; Rivat et al., 1976; Adetugbo et al., 1977). One of the earliest proposals was that the constant regions of immunoglobulin chains may be controlled by more than a single gene (Franklin & Frangione, 1971). Thus, residue 216 might be the beginning of a gene coding for the Fc fragment or, possibly, of a distinct gene for the hinge. Separate genetic control of the hinge is an attractive possibility since this portion of the heavy chain is not homologous with other domains (Edelman, 1970) and, because in certain immunoglobulins (IgG3 and IgA1), the hinge segment is replicated 2 or more times (Michaelson et al., 1977; Frangione & Wolfenstein-Todel, 1972; Liu et al., 1976).

Recent advances in our understanding of the organization of genes in eucaryotes have provided new insights into the

possible origin of immunoglobulin deletion variants. In a large number of cases, eucaryotic cistrons have been found to be discontinuous, with coding sequences (exons) interrupted by unexpressed or intervening sequences (introns); the mature messenger RNA is thought to be formed from the primary transcript by enzymatic excision of segments corresponding to the introns [see review by Crick (1979)]. For example, the constant and variable regions of mouse immunoglobulin κ and λ chains are encoded by noncontiguous segments of germline DNA; moreover, somatic rearrangement of the DNA appears to occur during the differentiation of antibody-forming cells (Hozumi & Tonegawa, 1976; Bernard et al., 1978; Brack et al., 1978; Lenhard-Schuller et al., 1978; Rabbits & Forster, 1978; Seidman & Leder, 1978; Seidman et al., 1978; Tonegawa et al., 1978).

Of particular relevance to the deletion in the Dob immunoglobulin is the very recent finding that discrete DNA segments code for the constant region of a mouse $\gamma 1$ chain (Sakano et al., 1979). Initial studies, by R-loop mapping, indicated the existence of three separate coding regions. The nucleotide sequences at the ends of each of these regions were compared with the amino acid sequence of the $\gamma 1$ chain reported by Adetugbo (1978a). This comparison revealed that each of the coding regions corresponds very closely to one of the constant-region domains. Indeed, one of the exons appears to encode exactly those amino acid residues (positions 121–214) that are deleted from the $\gamma 1$ chain in the IF2 variant of MOPC 21 (see Other Immunoglobulin Variants). However, the nucleotide sequences corresponding to the end of the C_H1 domain and the beginning of the C_H2 domain did not account for about 13 amino acids in the hinge region. Additional DNA sequencing then revealed another small exon that corresponds to the hinge sequence. Allowing for differences between the hinge regions of the murine and human proteins, this exon also appears to correspond quite closely to the missing segment in the three human hinge deletion proteins. Early et al. (1979) have recently obtained evidence that two introns divide the DNA coding for the constant region of a mouse α chain into three approximately equal segments, possibly corresponding to the three constant-region domains. Nucleotide sequences were not reported, and it is not yet known whether, in this case, there is also a separate exon for the hinge region.

The presence of introns between segments of DNA coding for the immunoglobulin domains would be expected to promote recombination and could lead either to the addition or to the deletion of a complete domain. Errors in the somatic rearrangement of the DNA, or in the RNA splicing mechanism, could also result in deletion variants. The benefits to the organism of having its "genes in pieces" have been discussed (Gilbert, 1978). However, the plasticity required to facilitate rearrangement of genetic material may entail the risk of the occasional formation of anomalous gene products.

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