

## Chemical Characterization of the Peptic Fragment of Rabbit $\gamma$ G-Immunoglobulin\*

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**ABSTRACT:** The 5S peptic fragment (Pep-I) has been examined with respect to its reductive dissociation into subunits and their properties, the products of further fragmentation with papain, and the number and location of the interheavy-chain half-cystines. As expected, Pep-I was shown by antigenic and amino acid analyses to contain the intact light chain. The other subunit, constituting a portion of the heavy chain, gave an amino acid composition significantly different from the corresponding papain fragment of the heavy chain (Fd). The presence of hexosamine in the peptic subunit confirms our previous conclusion that there are two carbohydrate moieties on the heavy chain. Digestion of Pep-I with soluble papain yielded a 3S fragment and a peptide fraction. The 3S product gave a molecular weight by equilibrium sedimentation of 39,000 ( $M_2$ ) which is very close to the value for the Fab fragment. The peptide fraction was equivalent to a fragment of mol wt 7000. Amino acid analysis of this fraction revealed no methionine and indicated, therefore, that Pep-I is smaller than the bivalent fragment resulting from limited cleavage of  $\gamma$ G with cyano-

gen bromide. The distribution of half-cystine and S-carboxymethylcysteine (S-CMC) between the 3S and peptide fractions, prepared in the presence of different concentrations of mercaptoethanol, showed no S-CMC in the 3S protein and an invariant sum of 2.0 moles for the contents of half-cystine and S-CMC/7000 g of the peptide. This is consistent with the presence of two interheavy-chain disulfides. Limited reductive cleavage of the whole  $\gamma$ G molecule and labeling of the resulting SH groups provided confirmation that the cleavage of a single disulfide was sufficient to separate the heavy chains. It was further concluded that there were no more than two interheavy-chain disulfides. The results also demonstrated that soluble papain cleaves the heavy chain on the N-terminal side of the interheavy-chain half-cystines whereas the closest point of peptic cleavage is on the C-terminal side.

The light-chain-containing fragments can be ordered with respect to size of the heavy-chain subunits as follows: CNBr > pepsin > water-insoluble papain > soluble papain.

In the original studies on the subunits of rabbit  $\gamma$ G-immunoglobulin (Fleischman *et al.*, 1963), from which the four-chain structure of the molecule was deduced (Porter, 1962), a model was proposed with five inter-chain disulfide bonds of which three cross-linked a pair of heavy chains. More recent studies by Palmer and Nisonoff (1964) and Hong and Nisonoff (1965) have demonstrated that in most  $\gamma$ G molecules the reductive cleavage of one interheavy-chain disulfide is sufficient to allow separation of the heavy chains from each other. This critical and labile disulfide also serves to link together the univalent fragments of the 5S product of the peptic digestion of  $\gamma$ G (Nisonoff and Dixon, 1964).

Further complexity has been added to the problem

of the number and reactivity of the interheavy-chain disulfides by the results of the digestion of rabbit  $\gamma$ G with water-insoluble papain. Whereas digestion with soluble papain in the presence of reducing agent yields univalent 3S fragments (Porter, 1959), treatment of  $\gamma$ G with water-insoluble papain, in the absence of a reducing substance, followed by prolonged exposure to 0.5% SDS<sup>1</sup> produces a 5S bivalent fragment (Cebra, 1964) similar to that arising from peptic digestion. In spite of their antigenic identity the two 5S fragments differed with respect to their molecular weight, that of the peptic product being significantly higher, and in their capacity to reoxidize to the original 5S form after conversion to the univalent form by mild reduction (Jaquet and Cebra, 1965).

The experiments with insoluble papain have thrown doubt on the conclusion, which may be drawn from the studies of Nisonoff and his associates noted above, that there is only one interheavy-chain disulfide in rabbit  $\gamma$ G. The doubt arises from the observation that digestion with insoluble papain of  $\gamma$ G (Cebra

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<sup>1</sup> Abbreviations used: SDS, sodium dodecyl sulfate; S-CMC, S-carboxymethylcysteine; Fr, fraction; SDeS sodium decyl sulfate.

*et al.*, 1961) and of the 5S peptic fragment (Jaquet and Cebra, 1965) did not change the molecular weight in either case. If there were only one disulfide bond then such a decrease should have occurred since peptide cleavage of the heavy chain had taken place. This cleavage was evident from the subsequent behavior on reduction of the digested  $\gamma$ G (Cebra *et al.*, 1961) and the digested 5S peptic fragment (Mage and Harrison, 1966).

The clarification of the structural relationship of the fragments resulting from the digestion of  $\gamma$ G with pepsin and papain, both soluble and insoluble, requires further information about the location of the sites of attack by these enzymes on the heavy chain. That the points of cleavage may differ has been recognized by several investigators particularly with respect to the difference between pepsin and soluble papain (Nisonoff and Dixon, 1964; Jaquet and Cebra, 1965; Utsumi and Karush, 1965). The clearest evidence for this difference is provided by the higher molecular weight of the peptic fragment (Jaquet and Cebra, 1965; Utsumi and Karush, 1965) and by the presence of a carbohydrate moiety on this fragment and its absence in the papain fragment Fab (Utsumi and Karush, 1965). A recent study by Inman and Nisonoff (1966) has indicated that the initial cleavage by soluble papain results in an Fc fragment with an interchain disulfide and that further action by the enzyme appears to remove a peptide containing this disulfide.

The experiments described in the present report were designed to clarify the number and location of the interheavy-chain disulfides of rabbit  $\gamma$ G-immunoglobulin. Particular attention was directed to the characterization of the 5S peptic fragment (Pep-I)<sup>2</sup> and to the analyses of the fragments resulting from the digestion of Pep-I with soluble papain. Since all of the original interheavy-chain disulfides appear to be retained intact in the 5S Pep-I fragment, these analyses and related experiments have served to provide partial resolution of the problems posed.

## Materials and Methods

**Preparation of Purified Rabbit  $\gamma$ G-Anti-Lac Antibody.** Antihapten antibody specific for the Lac group (*p*-azophenyl  $\beta$ -lactoside) was purified from a pooled antiserum by a method previously described (Utsumi and Karush, 1964) involving specific precipitation with Lac-human serum albumin and solubilization of the precipitate with lactose. The product consisted only of a 6.5S immunoglobulin which contained two immunochemically distinguishable components. These represent heavy-chain variants which are apparent by immunoelectrophoresis (Utsumi and Karush, 1965). The preparation was also antigenically heterogeneous with respect to its light chains (two components) as was also found in another rabbit antihapten antibody (Fujio and Karush, 1966). All of the protein in the

antibody preparation was antihapten antibody as judged by its binding capacity for hapten.

**Preparation of Normal Rabbit  $\gamma$ G-Immunoglobulin.** This material was isolated from the same pooled antiserum, after the removal of the anti-Lac antibody, by salt fractionation and chromatography with DEAE-cellulose as previously described (Utsumi and Karush, 1965). The antigenic and sedimentation properties of the normal  $\gamma$ G were the same as those of the antibody except that a third minor antigenic component was noted in the light-chain population of the normal  $\gamma$ G.

**Papain Digestion of 4.5S Pep-I.** Pep-I, the 4.5S product of the peptic digestion of  $\gamma$ G (Utsumi and Karush, 1965), was incubated at a concentration of 1.5% with papain (mercury complex, two times crystallized, Worthington) in 0.01 M acetate buffer (pH 5.5) and 0.001 M EDTA at an enzyme-substrate ratio of 1:100. Several concentrations of mercaptoethanol were included:  $14 \times 10^{-3}$ ,  $1.4 \times 10^{-3}$ , and  $0.14 \times 10^{-3}$  M, and the reaction was carried out for 24 hr at 37° in an atmosphere of nitrogen. At the lowest mercaptoethanol concentration, the papain was preincubated with  $1.4 \times 10^{-3}$  M of the reducing agent for 1 hr at pH 5.5 under nitrogen.

Subsequent to the digestion the reaction mixtures were alkylated with fresh solutions of iodoacetic acid (two times recrystallized), added under nitrogen, yielding a final concentration ranging from 0.02 to 0.005 M and sufficient to provide an excess over the mercaptoethanol. Following the addition of the iodoacetate solution the pH was increased to 8.2 with a solution of NaOH. The mixture was kept at room temperature for 2 hr and in the cold room overnight.

Portions of a preparation of Pep-I were also exposed to the same reduction and alkylation conditions as above but without the presence of papain. These products were subjected to the same fractionation procedure, to be described below, that was used for the papain-treated Pep-I.

**Reduction and Alkylation.** The  $\gamma$ G-immunoglobulin and Pep-I were also reduced and alkylated under conditions designed to split all of the interchain disulfide bonds. This was accomplished by the use of 0.14 or 0.28 M mercaptoethanol and incubation at pH 8.0 for 2 hr at room temperature. The alkylation was done with iodoacetate at pH 8.2. For the complete reduction of the disulfides the cleavage step was carried out with 0.56 M mercaptoethanol in 10 M urea followed by alkylation with iodoacetate.

**The Subunits of Pep-I.** These were isolated by the same procedure employed for the corresponding separation of the subunits of the papain fragment Fab (A and B pieces; Fleischman *et al.*, 1963). The subunits of Pep-I which were employed for amino acid analysis were obtained by a slightly modified procedure. A preparation of Pep-I which had been reduced with 0.14 M mercaptoethanol and alkylated was further treated with 0.14 M mercaptoethanol for 15 min in the presence of 4 M urea. The reaction mixture was cooled to 4° and the pH was lowered to 3.5 with concentrated

propionic acid. This step was followed by dialysis against 1 N propionic acid at 4° for 2 days. The protein remained soluble during these steps.

The fractionation of the mixture was done in 1 N propionic acid at 4° with a column (3.5 × 120 cm) of Sephadex G-75 using 40 mg of protein in 5 ml. A typical gel filtration pattern is shown in Figure 1 in which the aggregated heavy-chain fragment occurs in fraction I. The corresponding fractions from several runs were pooled.

**Molecular Weight Determination.** The molecular weight of the 3S component resulting from the papain digestion of Pep-I was measured by the method of sedimentation equilibrium in a short column (Van Holde and Baldwin, 1958). A column of 2.2 mm was used in a 12-mm double-sector cell with a filled-Epon centerpiece. Equilibrium was established at 20° and 13,410 rpm with the AN-D rotor of the Spinco Model E ultracentrifuge. The same value of  $\bar{v}$ , 0.730, was used as had previously been found for Pep-I (Utsumi and Karush, 1965).

**Analytical Ultracentrifugation.** Sedimentation experiments were carried out in a Spinco Model E analytical ultracentrifuge. The usual solvent was 0.2 M NaCl and 0.01 M sodium phosphate buffer (pH 7.8).

**Analysis of N-Terminal Amino Acid.** The 3S component resulting from the papain digestion of Pep-I was analyzed for N-terminal amino acids according to Porter (1957) using the method of Sanger.

**Amino acid analysis** was carried out in the Technicon Auto-Analyzer by the procedure of Spackman *et al.* (1958). The buffer system and elution procedure employed (Technicon Research Bulletin No. 10) were satisfactory, when used at 45°, for the separation of S-CMC from aspartic acid. The calibration of the

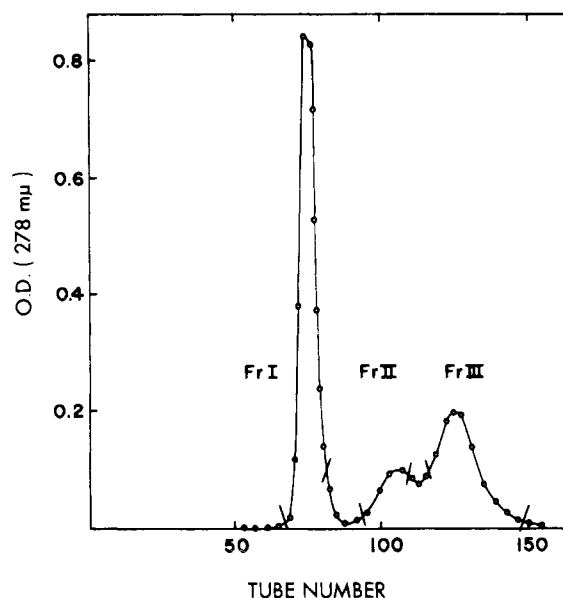


FIGURE 1: Fractionation of reduced and alkylated Pep-I into subunits by gel filtration with Sephadex G-75 in 1 N propionic acid at 4°. Fr I was Pep-IA and Fr III was Pep-IB.

analyzer was made with a standard mixture of amino acids (Technicon) containing 0.2  $\mu$ mole of each amino acid to which were added 0.20  $\mu$ mole of L-norleucine, 0.10  $\mu$ mole of L-S-carboxymethylcysteine, and 0.25  $\mu$ mole of glucosamine hydrochloride. The ratio of S-CMC to aspartic acid in the calibration mixture was similar to that observed in the experimental sample. Measured samples ranging from 2 to 12 mg were hydrolyzed in the usual way.

**Determination of Protein Concentration.** Protein concentrations were measured either by micro-Kjeldahl analysis for nitrogen or by optical density at 280 m $\mu$  with a Zeiss spectrophotometer. The optical method was used only when the ratio of optical density at 280 m $\mu$  to that at 250 m $\mu$  was not less than 2. These measurements were usually made in 0.04 M sodium decyl sulfate (pH 8) to assure complete disaggregation of the protein.

The extinction coefficients of the various proteins shown in Table I were determined by the combination of Kjeldahl analysis and optical density with the nitrogen content obtained by amino acid analysis.

**Determination of S-CMC Groups with [1-<sup>14</sup>C]-Iodoacetic Acid.** In some experiments the number of disulfide bonds reduced was measured by radioisotope assay with [1-<sup>14</sup>C]iodoacetic acid. A five- or tenfold dilution with nonradioactive iodoacetic acid (two times recrystallized) was made of [1-<sup>14</sup>C]iodoacetic acid (1.4 or 2.1 mc/mmole, New England Nuclear Corp.) and the diluted solutions were used for the alkylation of the sulfhydryl groups of the reduced protein. The counting was done with a Tri-Carb scintillation spectrometer, Model 3002. Measured volumes (0.1–0.2 ml)

TABLE I: Extinction Coefficients of Rabbit  $\gamma$ G-Immunoglobulins and Derivatives in the Presence of Sodium Decyl Sulfate.

Proteins <sup>a</sup>	$E_{1\text{cm}}^{1\%}$ in 0.04 M SDeS (pH 8)
Normal R $\gamma$ G	14.2
Anti-Lac antibody	14.2
Pep-I of R $\gamma$ G	15.0
Pep-I of anti-Lac	14.4
Pep-IA of R $\gamma$ G	15.5
Pep-IB of R $\gamma$ G	13.4
Pap(Pep-I)-I of anti-Lac	15.6

<sup>a</sup> Normal R $\gamma$ G was prepared from the same pooled serum from which the anti-Lac antibody had been removed. A nitrogen content of 16% was used for R $\gamma$ G and anti-Lac antibody. The values of 15.5 and 15.6% were determined for Pep-I and Pap(Pep-I)-I, respectively. Pep-IA: A piece from Pep-I; Pep-IB: B piece from Pep-I; Pap(Pep-I)-I: 3S component obtained by papain digestion of Pep-I.

TABLE II: Amino Acid Composition of Subunits of Pep-I.<sup>a</sup>

	Fr I (Pep-IA) (mol wt 24,000)			Fr II (mol wt 24,000) 24 hr	Fr III (Pep-IB) (mol wt 22,000)		Light Chain <sup>a</sup> Found
	23 hr	90 hr	Found <sup>c</sup>		24 hr	<sup>b</sup>	
Aspartic acid	14.7	14.3	14.5	14.7	17.1	(18)	18
Threonine	31.0	30.0	31.4 <sup>d</sup>	26.4	26.0	(27)	28
Serine	28.1	27.3	28.4 <sup>d</sup>	26.6	21.2	(22)	22
Glutamic acid	15.6	15.6	15.6	15.0	20.4	(21)	21
Proline	16.6	18.0	18.0 <sup>e</sup>	19.5	11.3	(12)	12
Glycine	21.2	20.8	21.0	21.5	18.1	(19)	19
Alanine	14.3	13.9	14.1	13.5	14.5	(15)	15
Valine	19.6	21.0	21.0 <sup>e</sup>	18.0	17.5	(18)	20
Half-cystine	4.1	3.9	4.0	4.9	5.9	(6)	6
S-CMC	2.7	2.8	2.8	2.4	0.9	(1)	1
Methionine	1.4	1.4	1.4	1.5	0.8	(0.8)	0.6
Isoleucine	5.2	5.4	5.3	4.8	5.9	(6)	6
Leucine	15.5	15.1	15.3	15.9	10.5	(11)	11
Tyrosine	8.9	9.0	9.0	7.1	9.7	(10)	11
Phenylalanine	6.4	6.4	6.4	6.0	5.8	(6)	6
Ammonia	22.8	25.0		22.5	23.7		
Lysine	9.2	9.2	9.2	9.2	8.7	(9)	9
Histidine	2.0	1.7	1.9	1.6	1.3	(1.4)	1.5
Arginine	5.7	5.8	5.8	5.3	3.2	(3)	3
Tryptophan							2
Hexosamine	0.72 <sup>f</sup>			0.4	0.0		
Mass recovery	23,300	23,400		22,700	21,300	(22,400)	22,400

<sup>a</sup> Data from Fleischman *et al.* (1963). <sup>b</sup> Multiplied by 22,400/21,300 for the comparison with the light chain.

<sup>c</sup> Estimation from 23- and 90-hr hydrolysis as the average or (d) by the extrapolation to zero hydrolysis time and (e) from 90-hr hydrolysis as the maximum recovery. <sup>f</sup> Uncorrected. <sup>g</sup> Moles of residue per mole of protein.

of the labeled protein solutions were dried on filter disks (23-mm diameter, Whatman 3MM) which were placed in glass vials containing 5 or 10 ml of phosphor-toluene scintillator solution. Nonreduced protein controls treated with the radioactive iodoacetic acid were included in the assays.

**Antisera.** Goat antisera were prepared with the light chains, heavy chains, and Pep-I of rabbit  $\gamma$ G used as antigens. An equine antiserum against rabbit  $\gamma$ G was also prepared. A goat antiserum against the Fab fragment of rabbit  $\gamma$ G was kindly provided by Dr. Mel Cohn. Antiallotype antisera were contributed by Dr. Sheldon Dray and Dr. H. Fujio who originally obtained them through the generosity of Dr. S. Dubiski. An attempt was also made to prepare goat antiserum against the heavy-chain fragment of Pep-I (Pep-IA) by immunization with Pep-IA of purified anti-Lac but no antibody could be detected.

## Results

**The Subunits of Pep-I.** The separation of the subunits of reduced and alkylated Pep-I from normal rabbit  $\gamma$ G-globulin was achieved by gel filtration with Sepha-

dex G-75 in 1 N propionic acid after exhaustive dialysis against this solvent and is illustrated in Figure 1. The first peak emerged at the void volume of the column indicating that this component was highly aggregated. The second and third peaks appeared at positions corresponding to those of the univalent Pep-I (3.5S) and the light chain, respectively. Repeated runs with the same preparation yielded average recoveries of the initial optical density of 54% in Fr I, 14% in Fr II, and 32% in Fr III.

Similar fractions were subjected to antigenic analysis by the Ouchterlony method with a variety of antisera. The fractions were first concentrated by ultrafiltration and dialyzed against neutral buffer. Samples of 2 mg of protein/ml were employed for gel diffusion. The proteins from the first peak (Fr I) exhibited virtually no reactivity except for trace precipitin lines in a few cases. These faint precipitin reactions were undoubtedly due to a minor contaminant in Fr I. The middle peak (Fr II) appeared to be a mixture of light chain and a material possessing antigenic groups associated with Pep-I. The presence of the former was inferred from the results with the anti-Pep-I and anti-Fab sera and the latter from the precipitin reaction with anti-Fab

serum absorbed with light chain. The third peak (Fr III) exhibited only the antigenic characteristics of the light chain and showed no reactivity with the absorbed antiserum. It appeared that the light chain was present in Fr III with its original antigenic structure intact.

When antiallotype sera were used the reduced Pep-I was found to contain all of the allotypic determinants tested (A1, A2, A3, A4, and A5). Among the three fractions only Fr III showed allotypic specificity, namely, A4 and A5. This is consistent with its identification as a light-chain population (Feinstein *et al.*, 1963). From the absence of allotypic reactivity in Fr I, which fraction is derived from the heavy chain, it can be inferred that its dissociation from the light chain has resulted in a conformational alteration accompanied by the loss of its allotypic specificity. Examination of Fr I and Fr III for N-terminal amino acids revealed less than 0.1 mole of alanine/46,000 g of Fr I and approximately 1 mole of alanine/46,000 g of Fr III. On the basis of these results Fr I and Fr III were identified as the subunits of Pep-I and were designated Pep-IA and Pep-IB, respectively. Fr II was considered to be a mixture of these subunits together with undissociated Pep-I.

Further characterization of the subunits of Pep-I was provided by amino acid analysis (Table II). Since Fr I became insoluble on aging it was dissolved in 0.04 M sodium decyl sulfate for measurement of protein concentration, and hydrolysis with 6 N HCl was done in the presence of the detergent. Subsequent chromatography of the hydrolysate was not affected by the presence of the detergent.

The amino acid compositions, shown in Table II, were calculated on the basis of assumed molecular weights of 22,000 for Fr III and 24,000 for Fr I and Fr II. The composition of Fr III (Pep-IB) is very close to that of the light chain (Fleischman *et al.*, 1963) shown in the last column when correction is made for the difference of molecular weight. Together with the results of the antigenic analysis, these results demonstrate that the subunit Pep-IB is an intact light chain. This behavior parallels the finding that an intact light chain can also be isolated from the fragments produced by papain digestion (Fleischman *et al.*, 1963). It may be noted that the figures for Fr III (Pep-IB) do not include corrections for loss during hydrolysis of threonine, serine, and tyrosine. The only significant discrepancy in the comparison with the light chain is the somewhat lower value for valine.

The presence of one S-carboxymethylcysteine and six half-cystine residues in Pep-IB provides verification for the earlier conclusion by Fleischman *et al.* (1963) that one disulfide bond links the light chain to the heavy chain and that the light chain of rabbit  $\gamma$ G possesses three intrachain disulfides.

The amino acid compositions of Fr I and Fr II are fairly similar to each other and quite different from that of Fr III (Pep-IB). The composition of Fr I (Pep-IA) is significantly different also from that of the heavy-chain fragment derived from Fab (Fleischman *et al.*, 1963). Of the three S-carboxymethylcysteines

of Pep-IA one is associated with the interchain disulfide of the light chain and at least one other must be attributed to an interheavy-chain disulfide. The third may arise from a second disulfide bond between the heavy chains but these analytical data are inadequate to establish this point.

The distribution of hexosamine is in accord with the previous findings (Utsumi and Karush, 1965) that Pep-I contained approximately 1 mole of hexosamine/mole of univalent fragment associated with the fragments of heavy chain present in Pep-I. Because of its low content an accurate determination could not be made in the amino acid analysis. However, if correction is made for 40% loss due to the acid hydrolysis and chromatography, all of the hexosamine found in Pep-I can be attributed to its heavy-chain fragment.

**Papain Digestion of Pep-I.** The further fragmentation of Pep-I by papain was investigated to provide clarification of the number and location of the interheavy-chain disulfide bonds. We have previously observed (Utsumi and Karush, 1965) that the treatment of the 4.5S peptic fragment of rabbit  $\gamma$ G-globulin with papain, by the procedure originally employed with the native  $\gamma$ G molecule (Porter, 1959), produced a 3S component and a group of small peptides. In the present experiments the 4.5S Pep-I of normal rabbit  $\gamma$ G and anti-Lac antibody were digested with papain in the presence of mercaptoethanol at concentrations of the reducing agent of  $1.4 \times 10^{-2}$ ,  $2.8 \times 10^{-3}$ ,  $1.4 \times 10^{-3}$ , and  $1.4 \times 10^{-4}$  M, followed by alkylation. Control reactions without the enzyme were also carried out.

The gel filtration patterns of one pair of reaction mixtures are shown in Figure 2. Fractionation was done on a column ( $3 \times 112$  cm) of Sephadex G-75 in 0.12 M ammonium acetate (pH 7.2) to separate the 4.5S and 3S components as well as the peptides. In Figure 2A, corresponding to a reaction mixture without papain, the first and second peaks represent the 4.5S and 3S proteins, respectively. The last peak contained only reagents since no amino acid was recovered from the acid hydrolysate of this fraction. In contrast (Figure 2B), little, if any, 4.5S protein survived the digestion in the presence of papain. The minor component at the 4.5S position (Figure 2B) was obtained only with the lowest mercaptoethanol concentration ( $1.4 \times 10^{-4}$  M). It was not characterized and consisted presumably either of undigested or aggregated protein. The value of  $s_{20,w}$  for the protein in the second peak obtained with papain was 3.26S at 0.4% protein concentration.

The complete series of experiments is summarized subsequently and the recoveries of the 4.5S and 3S proteins produced by simple reduction at various concentrations of mercaptoethanol are compared with those found after treatment with papain. In the absence of papain the extent of breakdown was dependent on the concentration of the reducing agent, reaching complete conversion only at a concentration exceeding  $1 \times 10^{-2}$  M. This is in accord with the original observations of Nisonoff *et al.* (1961) on the reductive cleavage of Pep-I. With papain the

TABLE III: Amino Acid Composition of Pep-I and Fragments Derived by Papain Digestion.<sup>a</sup>

	Whole Digest	Fr I (3S protein)	Fr II <sup>d</sup> (peptide)	I + II	Pep-I <sup>a</sup>
Analysis on 24-hr Hydrolysate					
Aspartic acid	32.1	27.1	4.7	31.8	31.5
Threonine	55.9	45.8	8.7	54.5	58.9
Serine	45.4	36.7	7.3	44.0	49.8
Glutamic acid	31.9	25.4	5.1	30.5	31.0
Proline	30.3	23.3	6.2	29.5	29.9
Glycine	39.1	32.9	6.1	39.0	39.1
Alanine	27.1	22.3	4.3	26.6	26.5
Valine	41.2	35.3	6.1	41.4	41.9
Half-cystine	11.9	11.6	0.9	12.5	14.2 <sup>b</sup>
S-CMC	1.5	0.0	1.1	1.1	
Methionine	2.2	2.1	<sup>c</sup>	2.1	2.2
Isoleucine	11.2	9.6	1.6	11.2	11.3
Leucine	26.8	21.8	4.6	26.4	27.3
Tyrosine	16.2	13.9	2.3	16.2	17.0
Phenylalanine	12.2	10.2	1.9	12.1	12.2
Lysine	18.4	14.6	3.2	17.8	18.2
Histidine	3.2	2.5	0.5	3.0	3.1
Arginine	10.0	8.4	1.6	10.0	9.8
Mol wt	46,000	39,000			46,000
Recovery (%)	96.3	93.2			

<sup>a</sup> Amino acid composition of Pep-I was evaluated from 24- and 48-hr hydrolyses in which threonine, serine, and tyrosine were extrapolated to zero hydrolysis time, and valine, isoleucine, and leucine were estimated from 48-hr hydrolysis. <sup>b</sup> Cystine content of Pep-I was estimated as S-CMC from the separate analysis of an extensively reduced and alkylated sample (see text). <sup>c</sup> Less than 0.1. <sup>d</sup> Calculated on the basis of the difference in molecular weight between Pep-I and the 3S protein (46,000–39,000) and the assumption that the total mass lost from Pep-I by papain digestion was fully recovered in Fr II. <sup>e</sup> Moles of residue per mole of fragment.

breakdown was independent of mercaptoethanol concentration down to  $1.4 \times 10^{-4}$  M.

The cleavage of Pep-I by papain in the absence of reducing agent was also carried out. For this purpose papain, at a concentration of 0.2%, was activated by incubation with 0.014 M mercaptoethanol and 0.002 M EDTA at pH 8 for 2 hr and the enzyme was isolated by gel filtration through a column of Sephadex G-25. The exposure of the 4.5S protein to the activated papain without reducing agent also led to the appearance of a 3S fragment and peptides. The recovery of the 3S product was over 80% after an incubation of 20 hr at 37° both at pH 7.0 and pH 5.5. In the fragmentation with papain and reducing agent the latter evidently served only to activate the enzyme which split off a peptide fraction containing the interheavy-chain half-cystines.

*Characterization of the Papain-Produced 3S Component.* For the measurement of the molecular weight of this component, Pep-I obtained from purified anti-Lac antibody was digested with papain in  $1.4 \times 10^{-3}$  M mercaptoethanol. The 3S fraction was concentrated, shown to be free of aggregation in the analytical

ultracentrifuge, and subjected to equilibrium sedimentation (Van Holde and Baldwin, 1958) at a concentration of 2.2 mg of protein/ml. In the analysis of the data the plot of  $(dn/dr)(1/r)$  vs.  $n$  showed slight upward curvature, indicating size heterogeneity of the sample. That this material does tend to aggregate in neutral buffer was indicated by an aggregate peak during repeated gel filtrations. The 3S protein itself, however, appeared homogeneous as judged by the symmetrical patterns seen both in gel filtration and in analytical ultracentrifugation. Thus the heterogeneity observed by equilibrium sedimentation was probably due to a small amount of aggregate.

The value of 40,800 for  $M_z$ , obtained using the entire range of equilibrium data, agrees with the molecular weight (40,800) reported for the papain fragment (Fab) prepared in the usual way and examined in a dissociating solvent, 8 M urea at pH 3.5 (Pain, 1963). When only the strictly linear portion of the above plot was used, comprising more than 50% of the column length, an apparent value of 39,000 was found for  $M_z$ .

In any case the resulting value of  $40,000 \pm 1000$  still agrees closely with the molecular weight of the

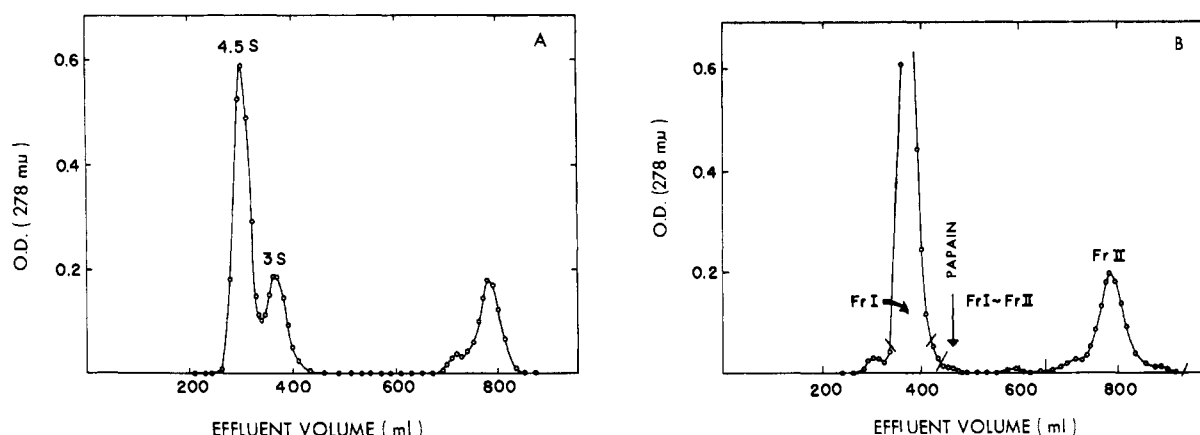


FIGURE 2: Gel filtration with Sephadex G-75 in 0.12 M ammonium acetate (pH 7.2) of reduced ( $1.4 \times 10^{-4}$  M mercaptoethanol) and alkylated Pep-I. (A) Without papain. (B) In the presence of papain.

Fab fragment resulting from papain digestion of the entire  $\gamma$ G molecule. It is substantially lower than the value of 46,000 for reduced Pep-I calculated from the results found with 4.5S Pep-I (Utsumi and Karush, 1965). It appears, therefore, that the papain digestion of Pep-I yields a macromolecular product which is very similar, if not identical with, the Fab fragment. A slightly more extensive breakdown in the former is not ruled out. The mass recovery of 85%, as judged by optical density, in the 3S product following papain digestion of Pep-I is consistent with the measured molecular weight of the product.

The immunochemical properties of the 3S component were evaluated with the following antisera: anti-Pep-I, anti-Fab, antilight chain, and antiallotypes. No differences were found when comparison was made by gel diffusion with Pep-I and Fab.

*Amino Acid Analyses of Pep-I of Anti-Lac Antibody and of the Fragments Produced by Papain Digestion.* The amino acid analyses of Pep-I of anti-Lac antibody and of the fractions obtained by digestion with papain in  $1.4 \times 10^{-3}$  M mercaptoethanol are shown in Table III. The analysis for Pep-I was evaluated from 24- and 48-hr hydrolysates except for the cystine content. For the accurate assay of this amino acid Pep-I was extensively reduced (0.56 M mercaptoethanol in 10 M urea) and alkylated. After hydrolysis for 24 and 48 hr, samples were analyzed for their content of S-carboxymethylcysteine.

The analyses of the papain digests were done both with the entire digests and with the separated fractions, Fr I (3S component) and Fr II (peptides), similar to those shown in Figure 2B. A third fraction, containing material collected between the two main fractions, was also analyzed. Both this fraction and Fr II were carefully freed of ammonium acetate and moisture before analysis. The results shown in Table III represent those found with fractions prepared under one set of conditions. The analysis of other preparations of the peptide fraction (Fr II) obtained from Pep-I of anti-

Lac antibody and  $\gamma$ G-immunoglobulin with several concentrations of reducing agent yielded compositions virtually identical with those listed in Table III.

The intermediate fraction contained only trace amounts of amino acids. On the basis of the quantity of Pep-I from which this fraction was obtained and after correction for the papain contribution, there were found maximally 0.6 mole of threonine, 0.4 mole of serine, 0.3 mole of glutamic acid, 1.1 moles of proline, and 0.3 mole of lysine per mole of Pep-I.

The amino acid compositions shown in Table III were calculated on the basis of molecular weights of 46,000 for Pep-I and the digest, 39,000 for the 3S component, and 7000 for the peptide fraction (Fr II), assuming quantitative recovery of the latter. The sum of the compositions of the two fractions agrees closely with that of the whole digest and is in satisfactory agreement with that of Pep-I when account is taken of the 24-hr hydrolysis period used for the fractions.

The absence of methionine in the peptide fraction indicates that pepsin cleaves the heavy chain between the interheavy-chain half-cystines and the nearest methionine toward the C-terminal end. Since limited cleavage with CNBr gives rise to a bivalent fragment similar to Pep-I (Cahnmann, *et al.*, 1966), it is likely that this fragment is somewhat larger than Pep-I. The absence of this methionine in Pep-I is also in accordance with our finding that of the total five to six methionines per heavy chain, three are found in Pep-III' and one in Pep-V (S. Utsumi and F. Karush, unpublished data).

The distribution of half-cystine and S-CMC is of particular interest in view of its relation to the question of the number of interheavy-chain disulfides. Although 1.5 moles of S-CMC was found in the whole digest (Table III), only 1.1 moles was recovered in the peptide fraction and none in the 3S fraction. Further analysis of the latter with a sample of 2.1 mg did not reveal any S-CMC. This discrepancy in the recovery of S-CMC

TABLE IV: Enzymatic and Reductive Fragmentation of 4.5S Pep-I.<sup>a</sup>

Prepn <sup>d</sup>	Concn of Mercapto-ethanol (M × 10 <sup>3</sup> )	pH/Papain	4.5S Component		3S Component		Peptides (moles)		Total S-CMC (moles)
			Yield (%)	S-CMC (mole)	Yield (%)	S-CMC (mole)	Half-cystine	S-CMC	
A	0.14	5.5/yes	2.6		97.4	0.0	1.9	0.15	0.2 <sup>b</sup>
A	1.4	5.5/yes	0		100	<0.1	0.9	1.1	1.5 <sup>b</sup>
N	1.4	5.5/yes	1.5		9.85	<0.1	0.4	1.5	1.8 <sup>b</sup>
N	1.4	5.5/yes	0		100	0.1	0.4	1.6	1.7 <sup>c</sup>
N	14	5.5/yes	0		100	0.6	0.0	2.0	2.6 <sup>c</sup>
A	0.14	5.5/no	72.7	0.0	27.3	<0.3			<0.1 <sup>c</sup>
A	0.14	8.0/no	27.6	0.05	72.4	0.6			0.4 <sup>b</sup>
A	1.4	5.5/no	14.7	0.1	85.3	0.7			0.6 <sup>c</sup>
N	1.4	5.5/no	8.0		92.0	0.75			0.7 <sup>c</sup>

<sup>a</sup> S-CMC contents of the proteins represent the number of residues/46,000 g for the simply reduced Pep-I and 39,000 g for the papain-digested protein, respectively. Half-cystine and S-CMC contents of the peptides are calculated on the basis of 7000 g of the total amino acids recovered. Total S-CMC was evaluated either by direct analysis of the unfractionated sample (b), or by calculation from the content of each component (c). <sup>d</sup> A is  $\gamma$ G anti-Lac antibody and N is normal  $\gamma$ G.

may be due to the inaccuracy of its assay in the whole digest because of its low content whereas its much higher content in the peptide fraction would allow a more accurate estimation. It is to be noted that the sum of half-cystine and S-CMC is 2.0 for the peptide fraction. However the reliability of this value is affected by the uncertainty in the molecular weight of the 3S protein.

The half-cystine content of the 3S fragment, about 12 moles/39,000 g, is in accord with previous analyses of the papain fragment (Fab) of  $\gamma$ G-immunoglobulin (Crompton and Wilkinson, 1963; Fleischman *et al.*, 1963) which showed 12 groups in piece II (42,000 g) and 13 as the sum for A and B pieces. The value of 14 for Pep-I in Table III is consistent with a value of 12 for the 3S protein and the value of 2.0 (half-cystine plus S-CMC) for the peptide fraction.

The content and distribution of cystine and S-CMC for a number of preparations are shown in Table IV. These include simple reductive cleavage as well as papain digestion at several concentrations of mercaptoethanol. It is evident that for the peptide fractions obtained by papain digestion of Pep-I the sum of half-cystine plus S-CMC remains invariant at 2 although their ratio varies widely over the range of experimental conditions. In addition no significant quantity of S-CMC was found in the 3S protein except when the highest concentration of mercaptoethanol was used,  $1.4 \times 10^{-2}$  M. In the case of reductive cleavage it is of interest to note that the surviving 4.5S molecule contained very little, if any, S-CMC. This implies that there was no intraheavy-chain disulfide bond susceptible to reduction under the conditions employed and that the splitting of one interchain bond was sufficient to bring about dissociation into 3.5S halves.

It is apparent from Table IV that the 3S protein formed by reductive cleavage contained substantially less than the expected one S-CMC group per half-molecule. This observation raised the possibility that the sulfhydryl group associated with the half-molecule was rendered unreactive toward iodoacetate because of conformational change following dissociation. This possibility was evaluated by the alkylation of reduced Pep-I in the presence of sodium decyl sulfate (SDeS). The protein (reduced with  $1.4 \times 10^{-3}$  M mercaptoethanol at pH 5.5) and a nonreduced control were treated with [1-<sup>14</sup>C]iodoacetic acid, first in the absence of SDeS for 2 hr at pH 8.2 and then followed by 16-hr incubation in the presence of 0.04 M SDeS. The carboxymethyl content of each sample was assayed at each stage by measurement of radioactivity. The results, shown in Table V, reveal that, although the uptake of

TABLE V: Analysis for Carboxymethyl Groups of Reduced and Alkylated Pep-I.<sup>a</sup>

	Without SDeS	With SDeS	S-CMC
Unreduced Pep-I	0.08	0.36	0.0 <sup>b</sup>
Reduced Pep-I	0.81	1.09	
Net <sup>c</sup>	0.73	0.73	

<sup>a</sup> Expressed as moles of carboxymethyl groups/46,000 g of protein. <sup>b</sup> Estimated by amino acid analysis.

<sup>c</sup> Carboxymethyl groups in the reduced alkylated sample corrected for the nonspecific groups in the unreduced protein.



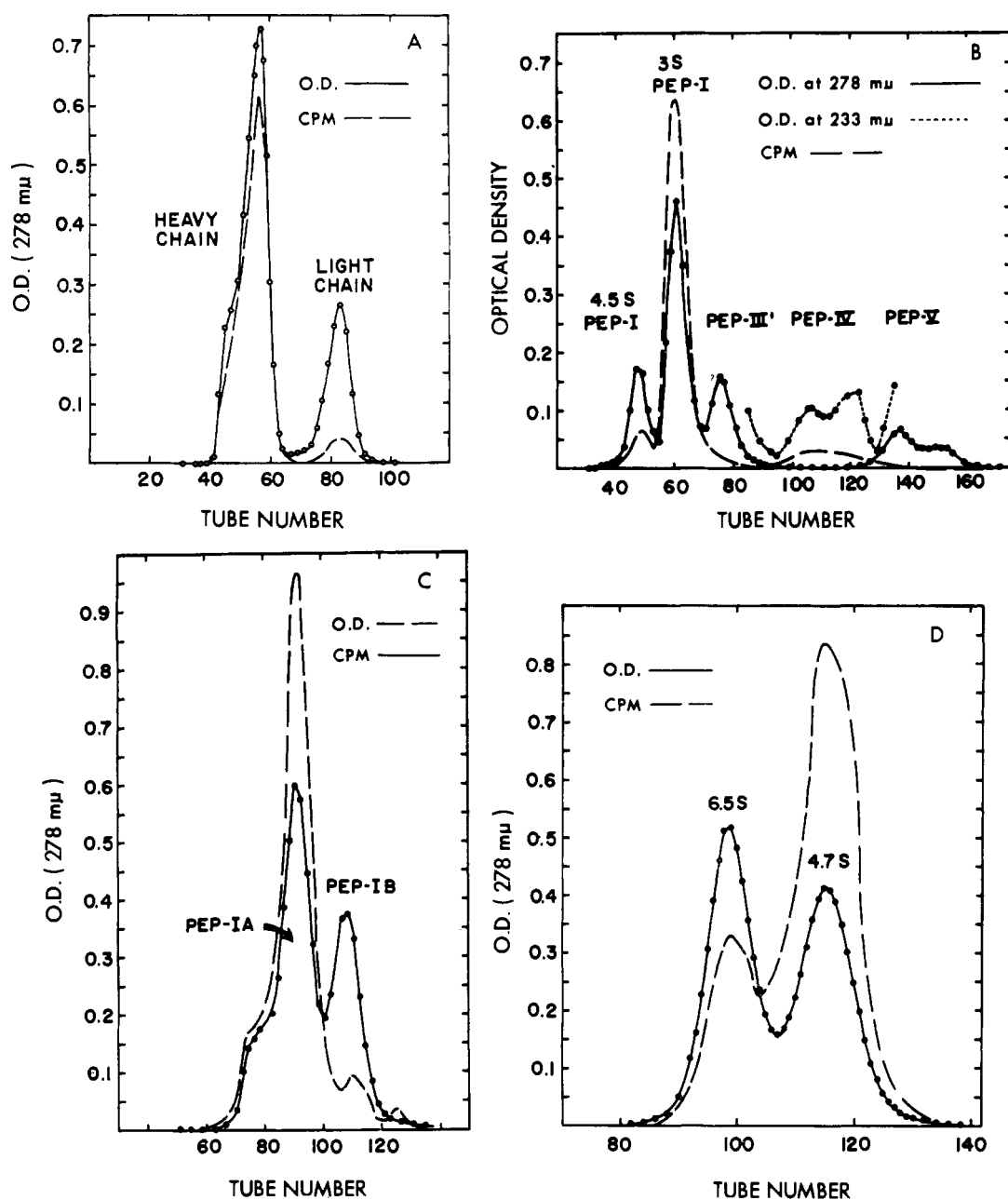


FIGURE 3: Fractionation into subunits and fragments using Sephadex G-75 after mild reduction and alkylation with [ $^{14}$ C]iodoacetate. (A) Separation of heavy and light chains in 1 N propionic acid. (B) Separation of peptic fragments in 0.2 M NaCl, 0.01 M sodium phosphate (pH 8.0), and 0.002 M EDTA. (C) Separation of subunits of Pep-I in 1 N propionic acid. (D) Separation into half and whole molecules of succinylated  $\gamma$ G in 0.2 M NaCl, 0.01 M sodium phosphate (pH 8.0), and 0.002 M EDTA.

carboxymethyl groups was increased by SDeS, there was no net increase in the number of S-CMC groups formed. The additional reaction facilitated by the detergent involved products other than S-CMC. The amino acid analysis with 1.5 mg of the sample of Pep-I treated with iodoacetic acid in 0.04 M SDeS also showed the absence of S-CMC groups. The failure to find the anticipated content of S-CMC in the half-molecule

was tentatively attributed to the formation of mixed disulfides with mercaptoethanol.

*Localization of the Interheavy-Chain Half-Cystines.* Nisonoff and his collaborators (Hong and Nisonoff, 1965; Nisonoff and Dixon, 1964) have demonstrated that the splitting of a single labile interheavy-chain disulfide in rabbit  $\gamma$ G-immunoglobulin will lead to the appearance of half-molecules in a dissociating

TABLE VI: Distribution of S-CMC Groups following Mild Reduction and Alkylation.<sup>a</sup>

	Original Molecule	Peptic Fragments			Polypeptide Chains		Subcomponents	
		4.5S Pep-I	3S Pep-I	Total Pep-I	A Chain	B Chain	Pep-IA	Pep-IB
Per mole of Unreduced	150,000 0.27	0.15	92,000	0.15	110,000 0.20	44,000 0.07 <sup>a</sup>	48,000 0.08 <sup>a</sup>	44,000 0.07 <sup>a</sup>
Experiment A <sup>c</sup>								
Recovery (%)		25.6	74.4	100				
CM groups	2.30	0.50	2.27	1.77	2.23	0.14	1.52	0.13
Net CM	2.03	0.35	2.12	1.62	2.03	0.07	1.44	0.06
Experiment B <sup>d</sup>								
					Succinylation			
					7S Molecule	Half-Molecule	Total	
Recovery (%)		53.5	46.5	100	53.0	47.0	100	
CM groups	1.42	0.38	1.63	0.96 <sup>a</sup>	0.70	2.16 <sup>b</sup>	1.39 <sup>a</sup>	
Net CM	1.15	0.23	1.48	0.81	0.43	1.89 <sup>b</sup>	1.12	

<sup>a</sup> Obtained by calculation. <sup>b</sup> Per mole of 150,000 mol wt. <sup>c</sup> Experiment A: the protein was reduced with 0.0028 M mercaptoethanol (moles of SH/mole protein = 33) for 24 hr at pH 5.5 and 37°. <sup>d</sup> Experiment B: the same preparation of protein as A was reduced similarly except the incubation temperature was 24°. <sup>e</sup> All alkylations were carried out with 0.007 M iodoacetic acid (<sup>14</sup>C labeled) for 3 hr at pH 8.2 and room temperature. Net carboxymethyl groups were calculated by the subtraction of the values found in the unreduced sample.

solvent. They have also concluded from the parallel behavior of 4.5S Pep-I that the same labile bond is involved in the reductive conversion of the 4.5S protein to the 3S Pep-I subunits. In the following experiments an attempt has been made to achieve a more detailed localization of the labile bond. This has been done by first converting the labile half-cystines to <sup>14</sup>C-labeled S-CMC groups followed by the preparation of various fragments and subunits from the labeled whole molecule.

The experiments were carried out with anti-Lac antibody which was reduced by exposure to 0.0028 M mercaptoethanol for 24 hr at pH 5.5 and 37° under nitrogen. The reduced protein was labeled by alkylation with 0.007 M [1-<sup>14</sup>C]iodoacetic acid for 3 hr at pH 8.2 and room temperature. After exhaustive dialysis one portion of the preparation was further reduced with 0.28 M mercaptoethanol and alkylated with nonradioactive iodoacetic acid. This material was used for chain separation by gel filtration with Sephadex G-75 in 1 N propionic acid. The fractionation pattern and the distribution of radioactivity are shown in Figure 3A.

A second portion of the labeled antibody was subjected to peptic digestion in the usual way (Figure 3B). One part of the isolated Pep-I (4.5 S + 3 S) was used for the separation of the 4.5S and 3S components. The other part was further reduced with 0.28 M mercaptoethanol, alkylated with nonradioactive iodoacetic acid, and fractionated by gel filtration (Sephadex G-75) in 1 N propionic acid to obtain the subcomponents Pep-IA and Pep-IB (Figure 3C).

The distribution of the radioactive carboxymethyl groups, introduced by the initial mild reduction and alkylation of the whole molecule, was measured in the preparations of heavy and light chains, the 4.5S and 3S Pep-I fragments, the Pep-IA and Pep-IB subcomponents, as well as the intact 7S molecule. The results are summarized in Table VIA in terms of the number of moles of carboxymethyl groups contained per mole of each species.

In another set of experiments half-molecules were prepared from labeled antibody by extensive succinylation and compared with the Pep-I components derived from the labeled and succinylated antibody. In this case the antibody was reduced and alkylated as above except that the incubation temperature for reduction was 24° instead of 37°. After removal of the reagents by passage of the reaction mixture through a Sephadex G-25 column, the protein solution (120 mg of protein in 20 ml of 0.2 M NaCl) was succinylated by the successive addition of 40-mg portions of solid succinic anhydride (mp 119–120°) to a total of 300 mg. The pH was maintained between pH 8.0 and 8.4 by addition of 2 N NaOH with a Radiometer titrator and the reaction period was 50 min.

A part of this preparation was fractionated with Sephadex G-150 using two columns (each 3.5 × 126 cm) in tandem in 0.2 M NaCl, 0.01 M sodium phosphate buffer (pH 8.0), and 0.002 M EDTA, and collecting 8-ml fractions at a flow rate of 64 ml/hr. Two peaks were observed, as shown in Figure 3D together with the radioactivity of each fraction, which sedimented

at 6.3 and 4.7S. No indication of separated light chain was seen in the analytical ultracentrifuge. The two fractions were antigenically indistinguishable from the native antibody although the precipitability of these materials was greatly reduced.<sup>3</sup> Another part of the succinylated protein was subjected to peptic digestion in the usual manner and the digest was fractionated on Sephadex G-75. The cleavage pattern was identical with that observed with similarly reduced and alkylated protein which was not succinylated, in terms of the recoveries of the 4.5S and 3S Pep-I components. The content of carboxymethyl groups in these preparations is shown in Table VIB.

The results of Table VI clearly show that the splitting of one interheavy-chain disulfide bond is sufficient to allow dissociation of most of the paired heavy chains, in confirmation of the conclusions of Nisonoff and his associates (Palmer *et al.*, 1963; Palmer and Nisonoff, 1964). The carboxymethyl (CM) groups introduced by the initial mild reduction and alkylation were almost entirely confined to the heavy chains. If correction is made for the slight extent of breakdown of the disulfide between the heavy and light chains, as measured by the figure of 0.06 for Pep-IB, the net uptake of CM groups in the formation of 3S Pep-I was 2.0/92,000. This result together with the fact that 78%<sup>4</sup> of the net CM groups present in the whole molecule and arising from the splitting of interheavy-chain bonds were recovered in the 3S Pep-I species provide the basis for the conclusion cited. Furthermore this recovery largely excludes the existence of an interheavy-chain disulfide bond on the C-terminal portion of the heavy chain, that is, outside of that part of the heavy chain corresponding to Pep-IA. This conclusion is further supported by the experiments with the succinylated antibody since the yields of the whole and half-molecules were identical with those of the 4.5S and 3S Pep-I components.

It may further be inferred, when these results are coupled with the analyses for the S-CMC content of the 3S component and the peptides formed by the papain digestion of 4.5S Pep-I, that the interheavy-chain half-cystines are localized in that portion of the heavy chain contained in Pep-I but removed from it by papain treatment. This conclusion is in agreement with earlier statements on this matter (Utsumi and Karush, 1965) and was further supported by separate experiments involving papain digestion of  $\gamma$ G-globulin. This was done in the presence of 0.0028 M mercaptoethanol at pH 7.0, followed by alkylation with <sup>14</sup>C-labeled iodoacetic acid. Neither Piece I or II (Porter, 1959) contained more than 0.1 mole of CM group/80,000 g of fragment.

In addition to the CM groups acquired by reduction

of the interheavy-chain disulfide the mild reduction reaction also resulted in the uptake of CM groups in the portion of the heavy chain other than Pep-IA. In expt A this was 0.41 CM group/whole molecule (2.03–1.62) and in expt B the value was 0.34 CM group (1.15–0.81). These were, in all likelihood, S-CMC groups. They appear to be associated with that portion of the heavy chain designated as Pep-IV (Utsumi and Karush, 1965), as may be seen in Figure 3B, since there was a significant content of radioactivity in the Pep-IV region. Analysis of Pep-IV-I has shown 2 moles of half-cystine residues/6500 g (monomer), which we associate with one intrachain disulfide. These results indicate that there is a relatively labile intrachain disulfide in this region of the heavy chain. It is reasonable to consider, therefore, that the third interheavy-chain disulfide assigned by Fleischman *et al.* (1963) may represent a susceptible intrachain disulfide bond in Pep-IV, although the presence of cysteinyl groups in this fragment remains a possibility.

It is seen in Table VI that the 4.5S Pep-I fragment acquired, as a consequence of reduction, 0.35 CM group in expt A (and 0.23 group in expt B). If correction is made for the splitting of the disulfide bond between the heavy and light chains the number is reduced to 0.23 in the heavy-chain fragment of the 4.5S Pep-I component. The relationship of the disulfide which gives rise to this 0.23 group, which is presumably S-CMC group, to the interheavy-chain half-cystines is not evident from the available data. In expt B the fact that the CM content of 3S Pep-I is only 1.48 rather than 2 is attributed to the formation of a mixed disulfide with mercaptoethanol.

## Discussion

Examination of the subunits derived from Pep-I by reduction and alkylation has established, as expected, that Pep-I includes the light chain in intact form. This conclusion was derived from the antigenic characteristics of Pep-IB, its allotypic specificity, and its amino acid analysis (Table II). Furthermore, the finding of approximately 1 mole of hexosamine/chain of Pep-IA supports our previous conclusion that there are two carbohydrate moieties on the heavy chain of  $\gamma$ G (Utsumi and Karush, 1965), the other one located on Pep-IV (part of Fc). The cleavage of the carbohydrate from Pep-I by papain has been observed by the recovery of a glycopeptide containing at least two-thirds of the Pep-I carbohydrate.

Following the papain digestion of Pep-I (4.5 S) a 3S component was isolated together with a fraction consisting of peptides. The 3S material appeared to be similar, if not identical, with the Fab fragment obtained by treatment of the original  $\gamma$ G-globulin with papain under the same conditions. Their similarity was evident by the agreement of their molecular weights and by their antigenic identity when examined by several different antisera. From the immunochemical behavior of the 3S component it could also be inferred that the intact light chain was contained in this component.

<sup>3</sup> We have also found that the reduction and alkylation of succinylated rabbit  $\gamma$ G, under the conditions commonly used for the preparation of heavy and light chains, lead to the spontaneous dissociation into heavy and light chains without the further addition of a dissociating agent (*cf.* Lenard and Singer, 1966).

<sup>4</sup>  $[(0.744)(2.12 - 2(0.06))]/[2.03 - 2(0.06)] = 78\%$ .

With this conclusion in mind a comparison was made of the amino acid composition of A piece (Fleischman *et al.*, 1963) and the amino acid analysis obtained by subtracting the peptide fraction (Fr II in Table III) from the heavy-chain fragment of Pep-I (Pep-IA in Table II). The amino acid analysis thus obtained by difference is that of the heavy-chain fragment contained in the 3S component. This comparison is summarized in Table VII. When the compositions are compared on the basis of equal molecular weights, it is evident that the A piece and heavy-chain fragment of the 3S material are very similar if not identical.

The analysis of the peptide fraction for its half-cystine and S-CMC contents (Table IV) bears on the question of the number of interheavy-chain disulfides. The fact that the sum of these figures was consistently 2, even though their ratio varied widely, points to the conclusion that there are two interchain disulfides in this portion of the heavy chain. It is conceivable that the value of 2 is a fortuitous figure arising from the heterogeneity of the peptide fraction with respect to its cystine content. Thus, if there were only one interchain disulfide and one-half of the peptide population also contained an intrachain disulfide, the same analytical result would be found. This possibility would be highly coincidental and does not find any independent support.

The analytical results concerning the content of half-cystine and S-CMC in the peptide fraction (Table IV) and the pattern of localization of CM groups following mild reduction and alkylation (Table VI) provide

evidence for the view that there may be two but no more than two interheavy-chain disulfide bonds. These bonds are localized in that portion of the heavy chain included in Pep-I but split off from it by papain treatment, *i.e.*, a C-terminal segment of molecular weight about 7000. The conclusion that there are no interheavy-chain disulfides in that portion of the chain excluded from Pep-I rests primarily on the observation that the separation of heavy chains was achieved with the introduction of only 0.2 CM group/C-terminal half (non-Pep-I) of the heavy chain. The absence of such disulfides was further indicated by the results with the succinylated antibody. In this instance the removal of the C-terminal halves of the heavy chain by peptic digestion did not affect the extent of heavy-chain separation following very mild reduction and alkylation.

Despite the apparent existence of two interheavy-chain disulfides, these chains can be separated by the cleavage of only one disulfide linkage. This observation, first made by Palmer and Nisonoff (1964), was confirmed by the results summarized in Table VI which showed that the reductive conversion of the 4.5S Pep-I to the 3S Pep-I involved the formation of only two sulfhydryl groups. It would appear, therefore, that the reduction of one interchain disulfide leads to a disulfide-interchange reaction which effects the cleavage of the second disulfide. We have previously proposed a possible mechanism for this process (Utsumi and Karush, 1965) but unequivocal experimental evidence for this scheme or any alternative is not yet available.

The present results serve to clarify the points of cleavage of the heavy chain by peptic digestion and treatment with soluble papain relative to the location of the interheavy-chain half-cystines. It has been previously suggested by several authors (Nisonoff and Dixon, 1964; Jaquet and Cebra, 1965; Utsumi and Karush, 1965) that these proteolytic enzymes break the heavy chain on opposite sides of the disulfides. The findings that the sum of half-cystine plus S-CMC is 2.0 for the peptide (Table IV) and the number of sulfhydryl groups acquired by 3S Pep-I is 1.0/monomer (Table VI) are quite definitive in this matter. They demonstrate that soluble papain cleaves the heavy chain proximal to the N-terminal end relative to the location of the interchain disulfides whereas the point of peptic cleavage nearest to this end is on the C-terminal side of these disulfides. Comparison of the peptic fragment with that formed by limited cyanogen bromide treatment (Cahnmann *et al.*, 1966) indicates that the latter is somewhat larger since the methionine which is converted to homoserine by this treatment is absent in the peptide formed by papain digestion of Pep-I.

It is of interest to note that water-insoluble papain behaves differently in this respect than the soluble papain. This is clearly seen in the results of the digestion of Pep-I by insoluble papain (Mage and Harrison, 1966). The resulting peptide, as indicated by its amino acid content, was much smaller in molecular weight than that found in this paper. Furthermore, the value of 1.1 groups of S-CMC in the peptide is consistent

TABLE VII: Amino Acid Composition of A Piece Derived from Pep-I.

	Papain A Piece from Pep-I (17,000 g)	Papain A Piece <sup>a</sup>	
		20,000 g	17,000 g
Aspartic acid	9.8	12	10.0
Threonine	22.7	27	22.5
Serine	21.1	25	20.8
Glutamic acid	10.5	12	10.0
Proline	11.8	15	12.5
Glycine	14.9	18	15.0
Alanine	9.8	12	10.0
Valine	14.9	19	15.8
Half-cystine	4.8	6	5.0
Methionine	1.4	1.3	1.1
Isoleucine	3.7	5	4.2
Leucine	10.7	14	11.7
Tyrosine	6.7	8	6.7
Phenylalanine	4.5	7	5.8
Lysine	6.0	7	5.8
Histidine	1.4	1.7	1.4
Arginine	4.2	5	4.2

<sup>a</sup> From Fleischman *et al.* (1963).

with our earlier suggestion (Utsumi and Karush, 1965) that water-insoluble papain splits the heavy chain between the pairs of half-cystines which link the heavy chains together. It may be concluded that the sizes of the heavy-chain portions of the various fragments containing the light chain may be ranked as follows: CNBr > pepsin > water-insoluble papain > soluble papain. It should be noted papain produces more extensive cleavage at pH 5.5, the condition used in the study, than it does at neutral pH.

Mage and Harrison (1966) have proposed an alternative interpretation, suggested earlier by Cohen and Porter (1964), to the idea of a pair of crossed disulfides (Utsumi and Karush, 1965) to account for the absence of a change in molecular weight after the treatment of  $\gamma$ G-globulin or Pep-I with water-insoluble papain. Their alternative is that there is a single interheavy-chain disulfide and that an intrachain disulfide spans the cleavage point in the heavy chain after digestion of  $\gamma$ G-globulin or Pep-I with insoluble papain. This bridge would serve to hold the Fab fragment to the remainder of the molecule. Reduction of the disulfide would then lead to the appearance of the Fab fragments and, in the case of Pep-I, a peptide. This model is rendered untenable by the present finding that the content of half-cystine plus S-CMC of the peptide resulting from digestion of Pep-I with soluble papain was 2 rather than 3 (Table IV). The latter value would be expected from their scheme since our 3S product did not contain any significant amount of S-CMC.

The pattern of cleavage of the heavy chain by soluble papain has been further illuminated in a recent report by Inman and Nisonoff (1966). It was found that digestion with papain for a short period, e.g., 1 hr, in the presence of 0.05 M L-cysteine gave rise to an Fc fragment which apparently retained at least one of the original interheavy-chain disulfides in reduced form. This was inferred from the capacity of the reduced Fc fragment to form an interchain disulfide by oxidation. On the other hand, a more extended digestion period, e.g., 10 hr, resulted in the loss of this property. It appears therefore that papain cleaves the heavy chain not only on the N-terminal side of the interchain disulfides but also, more slowly, on the C-terminal side. The isolation and analysis of the peptides resulting from the short and long digestion periods would probably shed additional light on the localization of the

interchain disulfides relative to the papain cleavage points.

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