Studies in Immunoglobulin Chemisty. I. Reinvestigation of the C-Terminal Amino Acids of 6.5S Rabbit and Human γ-Globulins*

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ABSTRACT: The C-terminal amino acids of rabbit and of human γ-globulin and of their heavy (H) polypeptide chains have been determined by hydrazinolysis. It was found that rabbit γ-globulin contains C-terminal glycine (2 moles), arginine (1 mole), and asparagine (1 mole), per mol wt 160,000; and that both glycines comprise the C-terminal ends of its H chains. In human γ-globulin, the C-terminal amino acids are glycine, arginine, cysteine (or half-cystine), and serine. The experimental data suggest integral values of 2 and 1 moles of glycine and arginine, respectively, per mole (160,000) of this immunoglobulin. Corrections applied from analyses of model polypeptides and from the urea-treated performic acid oxidized H chains allow for the estimation of approximately 0.5 mole of C-terminal cysteine or half-cystine and 0.5 mole of serine. Taken together, these data accord with the four-chain model currently accepted for γ-globulin. Since it was demonstrated that H chains from human γ-globulin also have only glycine at the C-terminal end, it can be hypothesized that C-terminal residues of immunoglobulin light (L) chains may be a species characteristic.

The nature of the C- and N-terminal amino acids of γ-globulin has been the subject of numerous investigations (reviewed by Eisen and Pearce, 1962; Porter and Press, 1962). In retrospect, most of these end-group determinations have failed to account satisfactorily, in terms of molar quantities, with the four-chain polypeptide structure (Fleischman *et al.*, 1962, 1963; Edelman and Poulik, 1961) which has emerged for IgG.[1] In one study, however, Silman *et al.* (1962) reported a total of 4.5 moles of C-terminal amino acids/mole of rabbit γ-globulin. They found glycine, serine, threonine, and alanine, in a 2:1:0.5:0.5 molar ratio in this immunoglobulin. The C-terminal amino acids of human γ-globulin were partially determined (Lay and Polglase, 1957) as glycine and serine, but more recent studies (Putnam *et al.*, 1963; Milstein, 1964; Titani and Putnam, 1965; Putnam and Easley, 1965) have suggested that cysteine occupies this position on some of the light (L) chains.

As part of a study of the amino acid composition of γ-globulins and antibodies, and of their fragments, the C-terminal amino acids of human and rabbit IgG were reinvestigated using the method of hydrazinolysis. The data that were obtained differed both qualitatively and quantitatively from those previously reported. Our results suggest that rabbit IgG contains C-terminal glycine, arginine, and asparagine in a 2:1:1 molar ratio (per mol wt 160,000), and that glycine occurs at the C-terminal positions of both heavy (H) chains. It was also found that the C-terminal amino acids of human IgG (prepared from Cohn fraction II) are 2 moles of glycine, 1 of arginine, and about 0.5 mole of serine. With oxidized human γ-globulin, approximately 0.5 mole of cysteine or half-cystine/mol wt 160,000 was found additionally as a C-terminal constituent. Here again, only glycine was revealed at the C-terminal positions of the H chains.

Experimental Section

γ-Globulin Preparations. Rabbit IgG was prepared from pooled rabbit γ-globulins (Pentex) by chromatography of 100-mg quantities, dissolved in 0.01 M KH_2PO_4–K_2HPO_4 buffer, pH 7.2, using DEAE-cellulose (80 × 2 cm) columns (Fahey and Horbett, 1959). Human γ-globulin (Cohen fraction II) was kindly provided by Dr. B. F. Erlanger. It was also resolved into its 6.5S components by chromatography on DEAE-cellulose.

Heavy Chains from γ-Globulins. A solution of 1 g of rabbit γ-globulin in 50 ml of 0.04 M KH_2PO_4–K_2HPO_4 buffer, pH 7.0, was treated under an atmosphere of nitrogen with 2-mercaptoethanol until the latter reagent reached a concentration of 0.1 M. After 2 hr the pH was adjusted to 8.0 with 1 N NaOH, and the mixture was made 0.2 M with recrystallized iodocetamide (Edelman *et al.*, 1963). After standing in the dark 10 min, the reaction mixture was dialyzed for 72 hr against three successive changes of 0.5 M propionic acid. The resultant H and L chains were resolved by chromatog-

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[1] IgG, γ-globulin, and 6.5S globulin are used interchangeably.

raphy on Sephadex G-200 using 0.5 M propionic acid as solvent (Fleischman *et al.*, 1963). Elution profiles of these proteins were followed by monitoring the effluent at 280 mμ. The H-chain fractions were divided into two parts, corresponding to the ascending and descending portions of its ultraviolet-absorbing elution profile. The combined fractions from the ascending portion of the curve were pervaporated at 8°, and the concentrated solutions were dialyzed for 72 hr against three successive changes of 0.5 M propionic acid. The contents of the dialysis bag were rechromatographed on Sephadex G-200. The eluent corresponding to the H-chain region of the chromatogram was dialyzed against running tap water for 48 hr, and the water was removed by lyophilization.

Quantitative chromatographic amino acid analyses of these H-chain preparations (Piez and Morris, 1960)[2] using the Technicon auto analyzer demonstrated the presence of the following free amino acids: aspartic acid, glutamic acid, serine, glycine, and half-cystine. These same free amino acids were also noted in the gel-filtration rechromatographed L chains obtained as purified by-products after H-chain resolution. It was not possible to remove these noncovalently linked, but strongly bound, amino acids from either H or L chains even by prolonged (96 hr) dialysis against water. To remove these contaminating amino acids, *ca.* 100 mg of an H-chain preparation was dissolved in 4.0 ml of 8 M urea and allowed to stand overnight. The solution was then dialyzed against tap water for 48 hr. The insoluble material was collected by centrifugation and was dried by lyophilization. Analysis by chromatography on Dowex 50-X8 demonstrated that this procedure removed adjunct amino acids. H-chain protein purified in this manner still exhibited its usual low solubility (*ca.* 10% in water at room temperature).

Performic Acid Oxidations. The oxidation of intact γ-globulin and of its H chains with this reagent was carried out by the procedure of Hirs (1956). The chilled, oxidized protein reaction mixture was transferred to a lyophilization apparatus and the water was removed by freeze-drying. The residue was redissolved in water and again lyophilized.

Hydrazinolysis. The experimental procedure described by Carlton and Yanofsky (1963) was used. Samples containing approximately 0.4 μmole of protein or of oligopeptide were dried *in vacuo* over P_2O_5 at 100° for 16 hr. They were then treated in heavy-walled 12-ml centrifuge tubes with 2.5 ml of anhydrous hydrazine, frozen, evacuated, sealed, and heated for 5 hr at 110°. The hydrazides were removed by precipitation with isovaleraldehyde. Following extraction with ethyl acetate, aliquots of the aqueous phase were subjected to amino acid analysis (Piez and Morris, 1960). Funatsu *et al.* (1964) have also used hydrazinolysis and direct automatic amino acid analysis to determine C-terminal residues of tryptic peptides from tobacco mosaic virus, but the steps involving derivative formation with isovaleraldehyde and extraction with ethyl acetate were omitted.

"Autoclaved" proteins were prepared for hydrazinolysis by treatment with 2 ml of distilled water in sealed centrifuge tubes for 2 hr at 110°. These samples were then dialyzed against water, lyophilized, and dried further *in vacuo* over P_2O_5.

C-Terminal Cysteine. Direct chromatographic analysis of free amino acids following hydrazinolysis and removal of hydrazides from oxidized human IgG showed an unresolved twinned peak in the cysteic acid region. One of these peaks was increased in size upon the addition of authentic cysteic acid to the reaction mixture. Combined paper electrophoresis–chromatography of the reaction mixture (Smith, 1960) revealed cysteic acid, plus several unidentified ninhydrin-positive spots which migrated in its proximity. The solutions containing presumptive C-terminal cysteic acid were therefore passed through a column of Dowex 2-X4 and the fraction which eluted with 0.1 N chloroacetic acid was collected (Schram *et al.*, 1954). After ether extraction and concentration of the water layer *in vacuo*, cysteic acid then appeared as a sharp peak at the correct elution volume upon analysis in the automatic amino acid analyzer.

Results

Using the model peptides leucylglycine, bradykinin, and hexapeptide B (containing the last six residues from the C-terminal end of bradykinin), the per cent recoveries of C-terminal glycine and of C-terminal arginine, following hydrazinolysis, were determined as 60 and 47%, respectively (Table I). In accordance with the findings of Niu and Fraenkel-Conrat (1955), C-terminal arginine appeared as ornithine. With both bradykinin and oligopeptide B, each of which contains a serine residue in its peptide sequence, approximately 10 mole % (corrected for destruction) of this amino acid appeared as a spurious C-terminal contribution. Examination of these two peptides by paper chromatography failed to reveal contamination with free serine. An oxidized sample of insulin, also chromatographically free of amino acids, gave a 61% yield of C-terminal alanine from its B chain, and also provided 22 and 18%, respectively, of extraneous glycine and cysteic acid.

When rabbit IgG was examined by hydrazinolysis and subsequent quantitative analysis (Table II), approximately 2 moles of glycine and 1 mole of arginine were found/mole (mol wt 160,000) of protein, in addition to the anticipated trace quantity of serine. Since the sum of these residues accounted for only 3 of the presumed 4 moles of C-terminal amino acid in γ-globulin, it was suspected that asparagine, which would not be detected in this method (Niu and Fraenkel-Conrat, 1955), might also be present.

Hydrolysis of rabbit IgG with steam under pressure, probably *via* the ammonium salt of resultant C-terminal aspartic acid, revealed this residue upon subsequent

[2] A starting citrate–sodium citrate buffer, pH 2.855, was used in the initial chamber of the Varigrad and the detergent was omitted.

TABLE I: Hydrazinolysis of Known Peptides and Oligopeptides.[a]

Amino Acid	Leu-Gly		Bradykinin[b]		Oligopeptide B[c]		Insulin	
	μmoles	% Recovery	μmoles	% Recovery	μmoles	% Recovery	μmoles[d] Found (cor)	% Recovery
Glycine	0.120	60					(0.022)	
Arginine			0.096	48	0.094	47		
Serine			(0.011)		(0.015)			
Alanine							0.061	61
Cysteic acid							(0.018)	

[a] Values represent an average from at least two determinations. Except for insulin (0.100-μmole samples), 0.200 μmole of each peptide was taken for analysis. [b] Sequence: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. [c] Sequence: Gly-Phe-Ser-Pro-Phe-Arg. We are indebted to Dr. R. Bircher, Sandoz, Inc., for supplying these two polypeptides. [d] This insulin sample was entirely free of contaminating amino acids, as judged by chromatographic analysis. Prior to hydrazinolysis, it was subjected to performic acid oxidation. Values in this and subsequent tables are corrected for recoveries as follows: C-terminal glycine, arginine (detected and measured as ornithine), and alanine, based upon data obtained from the model peptides as given above. Recoveries for aspartic acid, serine, and cysteic acid were found to be 48, 60, and 66%, respectively, by method of Niu and Fraenkel-Conrat (1955), in which a standard amino acid mixture was subjected to hydrazinolysis, derivatization, extraction, and automatic amino acid analysis, under reaction conditions identical with those used for the polypeptides. Figures enclosed in parentheses in this table indicate extent of contribution from non-C-terminal residues.

TABLE II: C-Terminal Amino Acid Analyses of Rabbit γ-Globulin and Its H Chains.[a]

Amino Acid	Sample Description			
	Rabbit γ-Globulin	"Autoclaved"[b] Rabbit γ-Globulin	H Chain	"Autoclaved"[b] H Chain
Glycine	0.374	0.382	0.188	0.212
Serine	0.039	0.041	—	—
Arginine	0.230	0.214	—	—
Aspartic acid	—[c]	0.196	—	—

[a] Results expressed as μmoles found (corrected as given in Table I)/0.200 μmole of protein taken for analysis. Assumed molecular weight for 6.5S γ-globulin, 160,000; assumed molecular weight for H chains, 50,000. Values given are the average of two determinations. [b] See Experimental Section for details. [c] Dash in table signifies less than 0.015 μmole was detected.

hydrazinolysis. The transformation of asparagine into aspartic acid with steam, or with hot lead hydroxide, has been noted in early work on these compounds (discussed in Greenstein and Winitz, 1961). There is no reason to believe that treatment with steam at 110° released any peptide-linked aspartic acid from within the protein chains (*cf.* Tsung and Fraenkel-Conrat (1965), who employed 0.03 N HCl), since chromatograms of H-chain preparations, after similar "autoclaving" and hydrazinolysis, did not reveal any detectable aspartic acid. In fact, hydrazinolysis and subsequent amino acid analysis both of unmanipulated and of "autoclaved" rabbit H chain demonstrated only the presence of 1 mole of glycine (per mol wt 50,000) in these preparations (see Table II).

Comparable analyses of human 6.5S γ-globulin yielded 2 moles of glycine, 1 mole of arginine, and approximately 0.5 mole of serine/mole (160,000) of this protein (Table III). In a separate experiment, no aspartic acid could be found following hydrazinolysis and chromatographic analysis of "autoclaved" human IgG. As was the case with rabbit γ-globulin, only C-terminal glycine was found in the human H chain (Table III). Since cystine and cysteine are destroyed by the procedure used (Locker, 1954; Niu and Fraenkel-Conrat, 1955), it was necessary to oxidize the protein to detect this C-terminal residue. The experimental data (Table III) demonstrated the presence of approximately 0.5 mole of cysteic acid/mole of intact human 6.5S γ-globulin, whereas none was found upon ex-

TABLE III: C-Terminal Amino Acid Analyses of Human γ-Globulin and Its H Chains.[a]

Amino Acid	Sample Description: Human γ-Globulin	"Oxidized" Human γ-Globulin	H-Chain[b] Preparation
Glycine	0.199	0.178	0.115
Serine	0.103	0.082	0.032
Arginine	0.106	0.109	—
Alanine	0.033	—	—
Cysteic acid	—	0.058[c]	—
Aspartic acid	—	—	—

[a] Results expressed as μmoles found (corrected according to Table I)/0.1 μmole of protein (6.5S preparation from Cohn fraction II). Assumed molecular weights 160,000 and 50,000, respectively, for human γ-globulin and its H chain. Except where otherwise noted, values represent average of duplicate experiments and determinations; a dash signifies none detected. [b] The purified H-chain preparation was also urea-treated and performic acid oxidized prior to hydrazinolysis. [c] A sample of 1.25 μmoles of urea-treated, performic acid oxidized human γ-globulin was subjected to hydrazinolysis and subsequent steps (see Experimental Section) to allow for unequivocal resolution of cysteic acid. This value represents a single determination on an aliquot of the final reaction mixture from this special large-scale experiment.

amination of performic acid oxidized H chains.

Discussion

The almost quantitative recovery of 4 moles of C-terminal amino acids/mole of 6.5S rabbit γ-globulins provides additional experimental evidence for the four-chain model of IgG presented by Porter (1963). Although our results for intact rabbit γ-globulin differ from those of Silman *et al.* (1962) to a great extent, their observation that glycine is essentially the only C-terminal amino acid in fragment III obtained from this immunoglobulin is confirmed by the present findings regarding the C-terminal residues of the H chains. In addition, it should be noted that Fleischer *et al.* (1961) earlier obtained evidence that implicated aspartic acid as the C-terminal amino acid in two specific rabbit antibodies. In most prior investigations, some variation of the hydrazinolysis method was employed. Several possible explanations for the discrepancies between the results given here and those previously reported can be offered. First, unless C-terminal asparagine is initially hydrolyzed to aspartic acid, it is lost during the procedure of hydrazinolysis. Second, because of passage of the products of hydrazinolysis through ion-exchange resins (Lay and Polglase, 1957), ornithine resulting from C-terminal arginine may have escaped detection. Third, it should be emphasized (Table I) that serine may appear in significant amount from the *endo* portion of a protein or polypeptide chain after hydrazinolysis. Finally, it should be recognized that considerable quantities of tightly bound amino acids are carried along with γ-globulins, even into the final stages of purification. This was exemplified by the difficulties that were encountered in attempting to free the H chain (see Experimental Section) from noncovalently linked amino acids which were potentially spurious contributors to C-terminal residues.

Lay and Polglase (1957) first demonstrated the presence of C-terminal glycine and serine in human γ-globulin and accounted for 0.5 mole of each. C-Terminal cysteic acid has been found in type I Bence-Jones proteins by several methods (Putnam *et al.*, 1963; Milstein, 1964; Titani and Putnam, 1965; Putnam and Easley, 1965; Hilschmann and Craig, 1965). The experimental findings herewith reported of 2 moles of glycine, 1 mole of arginine, and roughly 0.5 mole each of cysteine and of serine as the C-terminal residues of this immunoglobulin are qualitatively in accord with these results, since Bence-Jones proteins are considered to correspond to the L chains of some immunoglobulins.

The quantitative aspects of converting the experimental data for cysteine (or half-cystine) and serine (Table III) into moles of C-terminal residues per mole of IgG are subject to the following considerations. Since it was noted that the penultimate half-cystine in one of the interchain disulfide bridges of insulin gave rise to a spurious 18 mole % contribution of cysteic acid (Table I), this figure can be applied as a minimal correction to the amount of C-terminal cysteic acid experimentally found, which provides a corrected value of approximately 0.5 mole. In addition to finding spurious serine from model peptides (Tables I), hydrazinolysis of native and performic acid oxidized rabbit γ-globulin provided *ca.* 20 mole % of this residue as a C-terminal amino acid, and the oxidized H chain derived from the human γ-globulin sample gave rise to 32 mole %. Assuming that these quantities of serine arose as extraneous "C-terminal" contributions, a corrected value of about 0.5 mole of C-terminal serine for human γ-globulin can be calculated. These corrected values for C-terminal serine and cysteine are grossly in accord with the suggestions

of Milstein (1965) regarding the composition of the type I and type II light chains of human γ-globulins, and once again appear to fit better with the four- than with the six-chain model (Cohen and Porter, 1964) for human γ-globulin.

The foregoing findings indicate that the polypeptides which comprise the four chains of IgG, within a given species, may be relatively homogeneous with respect to their C-terminal amino acid residues despite the vast heterogeneity which is known to exist in this protein population. Putnam and co-workers (1965) have also suggested that the C-terminal portions of Bence-Jones proteins appear to be less variable than the NH_2-terminal regions. It is possible that half of the population of rabbit γ-globulin molecules contains arginine on both L chains, while the other half has two L chains with C-terminal asparagine. Nisonoff and Hong (1964) have presented arguments, based on indirect evidence, for such symmetry in individual γ-globulin molecules. The γ-globulin molecules might, by chance, be mixed in proportions that would give C-terminal asparagine and arginine in a 1:1 ratio. Alternatively, one L chain of a single rabbit γ-globulin molecule may end in arginine while the other ends in asparagine. In view of the well-known heterogeneity of 6.5S γ-globulins, both possibilities appear to us to be equally attractive. The situation with regard to heterogeneity of human γ-globulin L chains is even more complex. After subtracting glycine, which appears as the C-terminal residue of the H chain, the L chains of human γ-globulin contain three amino acids in the C-terminal position, arginine, serine, and cysteine in about 1:0.5:0.5 molar ratio.[3]

Since it was found that the H chains of both human and rabbit IgG immunoglobulins have the same C-terminal amino acid, glycine, it may be of interest to study the C-terminal amino acids of 6.5S γ-globulins and their respective polypeptide chains in other species. It is possible that the C-terminal amino acids of the L chain are species characteristic, while the corresponding amino acids of the H chains are specific for the type of immunoglobulin (γG, γA, or γM).

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[3] The presence of cystine or cysteine in rabbit γ-globulin in the C-terminal position is also a possibility, since performic oxidized rabbit γ-globulin preparations have not been studied by hydrazinolysis.