Amino Acid Composition of Univalent Fragments of Rabbit Antibody

Abstract. *Univalent fragments of γ-globulin preparations from four individual rabbits were fractionated and the amino acid compositions of end fractions were compared. For amino acids with uncharged side chains, differences were consistently less than one residue per fragment of molecular weight 50,000. There were differences in the content of amino acids, other than glutamic acid, with charged side chains; these were in the direction expected on the basis of relative strength of adherence to carboxymethyl cellulose and electrophoretic mobility. Exclusive of any possible differences in amide, the total difference in charged groups was three to four residues per fragment. Since the net charge of univalent fragments reflects that of the γ-globulin molecule from which the fragments are derived, corresponding differences in composition may also exist among whole γ-globulin molecules.*

The heterogeneity of rabbit γ-globulin molecules with respect to net charge has been demonstrated by analysis of reversal of boundary spreading in free electrophoresis (*1*), by zone electrophoresis (*2*), and by chromatography on Celite (*3, 4*) or ion-exchange cellulose (*5*).

There is a direct relationship (*6*) between the ease of elution from carboxymethylcellulose (CM-cellulose) of a rabbit γ-globulin molecule and the fragments, corresponding to Porter's fractions I and II (*7*), derived from it by treatment with papain. (We describe these fragments as "univalent" fragments, even when they are obtained from "normal" γ-globulin.) The existence of a parallel between the net charge of a rabbit γ-globulin molecule and that of its univalent fragments was thus suggested.

Our data indicate that there are differences in content of basic and acidic amino acid residues among univalent fragments and that these differences are consistent with their relative electrophoretic mobilities and strengths of adherence to CM-cellulose; the results support the hypothesis that 7*S* rabbit γ-globulin is heterogeneous in chemical composition.

While our work was in progress a note appeared by Feinstein (*8*) that reported differences in amide content among fractions of whole γ-globulin separated by electrophoresis, or univalent fragments derived from these fractions. If, as Feinstein assumed, the sum of dibasic acids and amide were constant among the fragments, differences in amide would correspond to differences in net charge. Our data indicate that there are small differences in the total of the free plus amidated dicarboxylic acids, and also, with respect to basic amino acids, between subfractions of the univalent fragments. These differences are in the direction expected on the basis of relative strengths of adherence to CM-cellulose and electrophoretic mobilities. The sum of these differences (three to four groups per fragment of weight 50,000) is comparable to the differences in amide.

Differences between subfractions of univalent fragments, with respect to each amino acid determined, which has an uncharged side chain, were less than one residue per fragment. The lower molecular weight of univalent fragments, as compared with whole γ-globulin, yields greater precision in terms of number of residues per molecule; this fact led us to investigate the composition of the fragments. The average standard deviation of replicate analyses, excluding amide, was equivalent to 0.23 residue per fragment. If the same precision were applied to whole γ-globulin, the average deviation would be approximately 0.7 residue per molecule and agreement to within one residue per molecule could not readily be established.

Tryptophan was not determined, and the data for cystine were not precise enough to be included in the comparisons, although no consistent trends were noted.

Similarities in the amino acid composition of purified rabbit antibodies of different specificities have been reported by Smith *et al.* (*9*), Fleischer *et al.* (*10*), and more recently by Koshland (*11*) who has also observed differences between antibodies of different specificity. Askonas *et al.* (*12*) have shown that fractions of γ-globulin which have been separated by electrophoresis are similar in composition.

Gamma-globulin preparations were made from the serums of each of four rabbits, one of which had been hyperimmunized with ovalbumin, by three precipitations with sodium sulfate (*13*) and passage through a column of diethylaminoethylcellulose (*14*). The sedimentation coefficients were 6.2 ± 0.2 *S*. Immunoelectrophoresis of two of the preparations gave single lines with goat anti-rabbit serum; two (rabbits N6 and N7) contained small amounts of a β-globulin. However, only γ-globulin was observed when paper electrophoresis was carried out. Corollary control experiments indicated that a contamination of 3 percent β-globulin would have been detected.

Gamma-globulin preparations were treated with 0.5 to 1.0 percent by weight of crystallized papain for 5 minutes at 37°C in 0.1*M* phosphate buffer, *p*H 7.5, containing 0.002*M* disodium ethylenediaminetetraacetic acid (EDTA) and 0.03*M* cysteine; samples were incubated with cysteine and EDTA for 1 hour at 37°C before papain was added. The release of small peptides, not precipitable by 5 percent trichloroacetic acid, was less than 8 percent of the total protein in each case. Several preliminary experiments showed that over 90 percent of the γ-globulin was degraded to 3.5*S* under these conditions. Ultracentrifugation was carried out after the preparations had been passed through CM-cellulose.

The papain was inactivated and small fragments were removed by passing the reaction mixture through a column of Sephadex G-50 equilibrated with 0.001-*M* sodium salt of *p*-chloromercuribenzoic acid (CMB) in 0.1*M* sodium acetate, or, in one case, by adding excess CMB directly to the reaction mixture and dialyzing repeatedly. Crystals of fraction III that formed were removed by centrifugation. The remainder of fraction III was removed by passage through a CM-cellulose column (0.01*M* acetate, *p*H 5.8); this step also removes undigested γ-globulin, which is adsorbed strongly under these conditions. The purified univalent material migrated in each case as a single peak (~3.5*S*) in the ultracentrifuge. A single line, characteristic of univalent fragments, was obtained by the method of immunoelectrophoresis in which a mixture of sheep antisera prepared against pepsin-treated (5*S*) rabbit γ-globulin and against 7*S* γ-globulin was used.

Subfractions of univalent material were obtained by stepwise elution from a CM-cellulose column. The size of the column was approximately $1 cm^3/mg$ of protein. The first fraction was obtained by eluting with 0.01*M* sodium acetate buffer, *p*H 5.1 (the protein was first dialyzed against the same buffer). A second fraction, roughly 50 percent

of the protein, was obtained by eluting with 0.01*M* sodium acetate buffer, *p*H 5.5, and a third by eluting with 0.1*M* sodium acetate, *p*H 5.4. In one case, the first two fractions were collected at *p*H 5.0 and 5.3, instead of 5.1 and 5.5. Amino acid analyses were run on the first and third fractions, each of which contained roughly a fourth of the total protein.

When paper electrophoresis was carried out at *p*H 8.6, the fraction eluted first from CM-cellulose moved more rapidly toward the anode in each case than the fraction eluted last, thus indicating a parallel between net charge and strength of adherence to CM-cellulose. End fractions were run on adjacent strips; samples from rabbits N6, N7, and Y2 were tested.

Samples to be compared were dialyzed together against 0.02 *M* sodium chloride, dried, and hydrolyzed (for 22 hours) in an evacuated sealed tube with 1.5 ml of constant-boiling HCl at 110° ± 0.2°C. Amino acid analyses were done by the procedure of Spackman *et al.* (*15*) with a Spinco automatic analyzer. In most instances, 1.3-mg samples were analyzed on each column. For a given sample the same amounts of material were delivered to the short and the long columns. Samples to be compared were always analyzed with the same batch of ninhydrin solution, and comparisons were made with the same set of values for a standard mixture of amino acids. Replicate analyses were carried out and samples to be compared were run alternately.

The results are reported in Table 1 as percentages of the total number of micromoles of amino acid present, as calculated from the analyses. The sum of the values for lysine through phenylalanine equals 100 percent. This eliminated the necessity for an accurate preliminary determination of the amount of protein being hydrolyzed. The latter was estimated with the extinction coefficient at 280 mμ, 1.48 optical density units per milligram per milliliter. We assume that tryptophan and cystine, which are omitted, account for 5 to 6 percent of the residues (*16*); small differences, if any, between fractions, with respect to tryptophan and cystine, would have a negligible effect on the calculated composition with respect to each of the other amino acids. The results for threonine and serine are somewhat low because of destruction during the 22-hour hydrolysis; however, samples to be compared were hydrolyzed under identical conditions. Methionine sulfone was not observed. Data for ammonia (Table 1) were corrected for the small amounts present in the diluting buffer and the hydrochloric acid used for hydrolysis, as estimated by analyses in the amino acid analyzer.

Results of analyses are given in Table 1. The number of replicate analyses performed on each sample is given in the heading. Values are tabulated for differences between end fractions with respect to each amino acid and calculated on the basis that the weight contribution of the amino acids analyzed, per mole of fragments, is 46,000 g. This assumes a molecular weight of 50,000, with an allowance of 8 percent for the contribution of cystine, tryptophan, and carbohydrate. If the correct value were, for example, 10 percent lower, *differences* in mole percent would be proportionately lower, but ratios would be unchanged. Analytical results are given for rabbit N6. Differences between end fractions are tabulated for each of the four rabbits, together with values of *P*. The latter values were calculated by a *t*-test to determine the significance of the difference between the means of the end

Table 1. Comparison of amino acid composition of chromatographic fractions of univalent fragments of rabbit γ-globulin. The number of replicate determinations (first and third fractions) were N6,3 and 3; N7,3 and 3; N12,2 and 2; Y2,2 and 3.

Average residues per molecule	Mole percent ± standard deviation, Rabbit N6		Δ Residues per molecule† and (*P*)‡			
4 rabbits*	1st fraction	3rd fraction	Rabbit N6	Rabbit N7	Rabbit N12	Rabbit Y2
			Lysine			
17.6	3.85±0.04	4.11±0.06	+1.2 (<0.01)	+1.4 (<0.01)	+1.1 (0.1)	+0.9 (<0.01)
			Histidine			
2.8	0.53±0.03	0.66±0.02	+0.6 (<0.01)	+0.6 (<0.01)	+0.6 (0.3)	+0.3 (<0.01)
			Arginine			
10.0	2.21±0.02	2.34±0.04	+0.6 (<0.01)	+1.0 (<0.01)	+0.3 (0.1)	+1.1 (<0.01)
			Aspartic acid§			
34.7	7.82±0.17	7.46±0.05	−1.6 (0.03)	−0.5 (0.4)	−0.8 (0.1)	−0.7 (0.03)
			Threonine			
63.3	14.28±0.10	14.38±0.04	+0.4 (0.2)	−0.8 (0.2)	+0.7 (0.5)	+0.3 (0.4)
			Serine			
51.2	12.06±0.13	11.99±0.06	−0.3 (0.4)	−0.2 —	−0.4 (0.6)	+1.1 (<0.01)
			Glutamic acid§			
35.4	8.05±0.08	8.09±0.02	+0.2 —	+0.4 (0.05)	−0.4 (<0.01)	−0.4 (0.1)
			Proline			
31.3	7.02±0.22	6.97±0.13	−0.2 —	−0.3 (0.2)	−0.2 —	+0.2 —
			Glycine			
42.7	9.89±0.06	9.75±0.05	−0.6 (0.04)	−0.8 (0.02)	0.0 —	−0.7 (0.04)
			Alanine			
31.0	6.90±0.04	7.05±0.06	+0.7 (0.03)	−0.4 (0.05)	+0.4 (0.3)	+0.2 —
			Valine			
43.9	9.86±0.07	9.98±0.04	+0.5 (0.05)	+0.2 —	−0.2 —	−1.1 (0.05)
			Methionine			
2.4	0.47±0.02	0.46±0.01	0.0 —	+0.2 —	−0.1 —	−0.2 —
			Isoleucine			
12.7	2.96±0.02	2.79±0.01	−0.8 (<0.01)	0.0 —	+0.1 —	−0.2 —
			Leucine			
29.4	6.54±0.03	6.55±0.07	0.0 —	−0.4 (0.1)	−0.4 (0.6)	+0.3 (0.2)
			Tyrosine			
20.2	4.62±0.09	4.60±0.07	−0.1 —	−0.3 (0.2)	−0.2 —	−0.9 (<0.01)
			Phenylalanine			
13.4	2.93±0.06	2.84±0.04	−0.4 (0.1)	−0.3 (0.06)	−0.5 (0.4)	−0.4 (0.2)
			Ammonia‖			
46.4	9.87±0.33	10.76±0.31	+3.9 (0.03)	−1.8 (0.05)	+0.2 (0.5)	+1.8 (0.6)
			Glucosamine‖			
2.2	0.47±0.01	0.44±0.03	−0.1 —	+0.9 (0.07)	−0.1 —	−0.2 —
			Galactosamine‖			
0.4	0.10±0.07	0.09±0.01	0.0 —	+0.6 (<0.01)	−0.3 (0.2)	+0.4 (0.1)

* Overall average of the first and third fractions (four rabbits). † Value for the third fraction minus that for the first fraction. ‡ The value, 1-*P*, is the level of confidence for the existence of a real difference between the means of the values for the first and third fractions (see text). Values of *P* are not given for differences less than 0.3 residue per molecule. § Total of free acid and amide. ‖ Number of micromoles in sample divided by the number of micromoles of amino acids (lysine through phenylalanine in the table) × 100.

fractions (*17*). The value, 1-*P*, gives the level of confidence for the existence of a real difference between the two mean values. Values of *P* are not included where the difference is less than 0.3 residue per fragment, since such small differences cannot be regarded as significant.

Consideration first of amino acids, other than amide, with uncharged side chains, shows that in almost every instance the difference between the end fractions is less than one residue per protein fragment. The average of all values of differences for the uncharged amino acids is 0.4 residue per fragment. The signs of the differences (value for the third fraction minus that for the first), among those amino acids, are consistent among the rabbits only for glycine and phenylalanine. The average differences are −0.6 residue for glycine and −0.4 for phenylalanine.

Consistent differences were, however, observed among the amino acids with charged side chains. These are in the direction expected on the basis of ease of elution from CM-cellulose. The fragments eluted most readily (first fraction) gave higher values for aspartic acid (total of free acid and amide) and lower values for lysine, histidine, and arginine. For rabbits N6, N7, and Y2 the confidence levels for the existence of a real difference were high [$(1-P)>0.95$], except for the aspartic acid of rabbit N7. Only duplicates were obtained for rabbit N12 and confidence levels are somewhat lower. Histidine is considered as having a charged side chain because the fractionation was carried out a *p*H below its *p*K. The signs of the small differences for glutamic acid were not consistent.

One can estimate the effect of these differences on the relative net charge of the fractions by summing up the differences for lysine, histidine, arginine, and aspartic acid (the sign for aspartic is reversed). The respective totals for rabbits N6, N7, N12, and Y2 are 4.0, 3.6, 2.8, and 3.0 residues per fragment of weight 50,000.

The fractions from different rabbits were analyzed with different batches of ninhydrin and compared with different standard curves. This results in some loss of precision. Although the values for the four rabbits were in good agreement, the possibility of small differences could not be excluded.

Feinstein's results (*8*) and our data indicate that there are differences in composition among molecules of rabbit γ-globulin with respect to amino acids having charged side chains. Koshland (*11*) has found differences in the amino acid composition of two specifically purified antibodies directed against a positively and a negatively charged haptenic group. The differences Koshland found also reside in amino acids having charged side chains; the relative electrophoretic mobilities of the two antibodies would be of interest in this connection. The agreement among other amino acids (*11*) was within the experimental error of one to two residues per molecule of weight 160,000.

The standard deviations of nearly all replicate measurements reported here were well under one residue per fragment. This is an obvious advantage of working with smaller pieces. A disadvantage, considered below, is the possibility of introducing artifacts by enzymatic digestion.

Our results strongly suggest that the separability of univalent fragments on CM-cellulose may be attributable to differences in amino acid composition. Our data for amide are probably less reliable than Feinstein's since we made no effort, aside from dialysis, to remove bound ammonia and because the decomposition of serine and threonine during hydrolysis increases the amount of ammonia present. However, fractions being compared were dialyzed together and handled identically. Our results neither confirm nor refute the report that there are four to five additional amide groups in the more basic fragments (*8*). However they do suggest that differences are small; the differences which we observed vary from −2 residues per fragment for rabbit N7 to +4 residues for rabbit N6.

Exclusive of amide, there are consistent differences of three to four residues per molecule between end fractions (Table 1). The results are reported in terms of the total of glutamic acid plus glutamine or aspartic acid plus asparagine. Any differences in amide must be superimposed on these values. If one accepts the difference as four residues of amide, then the total contribution to the difference in average net charge of the fragments would be seven to eight units. Since there is a parallel between the net charge of a γ-globulin molecule and that of its univalent fragments (*6*), corresponding differences in composition may account for the electrophoretic heterogeneity of γ-globulin.

Several factors argue against the possibility that differences in amino acid composition may actually result from contamination by fragment III of the last univalent fraction to be eluted. (i) The fragments were passed through CM-cellulose twice under conditions at which fragment III is ordinarily retained. (ii) Fragment III was not detected by immunoelectrophoresis. (iii) Despite its greater basicity, fragment III has slightly more aspartic acid per unit weight than the univalent fragments (*7*). (iv) Contamination should also have been reflected in the content of methionine, alanine, glycine, and isoleucine because the differences in composition between univalent fragments and fragment III, expressed as a ratio, are about as great for these amino acids as for the basic amino acids. No such trends were noted except for a slightly lower glycine content in the last univalent fraction eluted.

Some difference in charge may possibly reside in carbohydrate. However Feinstein reports (*8*) that differences in sialic acid content between end fractions of whole γ-globulin are not appreciable, and our data indicate that the glucosamine and galactosamine content of the univalent fractions do not vary significantly (*18*).

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References and Notes

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18. Supported by grants from the National Science Foundation and the National Institutes of Health.

19 March 1963