Location of Amino Acid Differences in the Subunits of Three Rabbit Antibodies*

Marian Elliott Koshland,† Frieda M. Englberger, and Roslyn Shapanka

ABSTRACT: Amino acid analyses have been carried out on the subunits of three rabbit antibodies isolated from animals homozygous for γ-globulin production. The characteristic differences in amino acid composition previously observed in the analyses of the whole antibodies were found to be distributed in both the heavy chain and the light chain subunits. For example, the serine and part of the tyrosine differences between anti-phenylarsonic acid antibody and anti-phenyl β-lactoside antibody were located in their respective heavy chains, while the remaining tyrosine and all of the aspartic acid differences occurred in the light chains. These results are consistent with present information that both chains contribute to the specificity of binding, and thus they provide further evidence that the observed differences in primary structure are associated with antibody reactivity. Small but significant differences were also found in the proline, valine, and alanine contents of the subunits. These had not been noted in the previous analyses of the whole antibodies either because the preparations assayed were of mixed allotypy or because a difference in an amino acid yield in one chain was obscured by a compensating change in another chain. The fractional recoveries obtained in the light chain analyses showed that the preparations from each antibody consisted of at least two types of chains which differed slightly from each other in their amino acid content. These data provide a chemical basis for the heterogeneity of light chains demonstrated in studies of their electrophoretic mobilities, antigenic properties, etc. A hypothesis that the light chains of a given antibody contain two regions identified with specificity, one of invariant and one of variable amino acid composition, is proposed to explain the dual findings of a characteristic average amino acid composition and a multiplicity of structure.

Recent work in our laboratory has demonstrated characteristic amino acid compositions for three rabbit antibodies, one directed against the negatively charged phenylarsonic acid group, one directed against the positively charged phenyltrimethylammonium ion, and one directed against the uncharged phenyl β-lactoside moiety (Koshland and Englberger, 1963; Koshland *et al.*, 1964). In comparison with anti-phenylarsonic acid antibody, the anti-phenyltrimethylammonium antibody was characterized by a higher aspartic acid and leucine content and by a lower arginine and isoleucine content, while the anti-phenyl β-lactoside antibody was characterized by a higher aspartic acid and a lower serine and tyrosine content. Since these amino acid differences were also observed when the antibodies were prepared in rabbits homozygous for γ-globulin production, they could not be attributed to the concentration of the antibodies into known genetic types of γ-globulin. Furthermore, the probability that each of the three antibodies was reproducibly isolated from a separate pool of differently charged γ-globulin molecules was found to be negligible. The best interpretation of the data, therefore, is that the antibodies have differences in primary structure which are associated with their immunological specificities.

To obtain additional evidence for this interpretation, studies were undertaken of the location of the amino acid differences in the subunits of the three antibodies. The extensive data of Edelman (1959), Edelman and Poulik (1961), Porter (1962), and Fleischman *et al.* (1962, 1963) indicate that the γG immunoglobulin of various species has a four-chain structure consisting of two pairs of polypeptide chains. One type, the heavy or A chain, has a molecular weight of 50,000–55,000 and the other, the so-called light or B chain, has a molecular weight of 20,000–25,000 (Pain, 1963; Small *et al.*, 1963; Marler *et al.*, 1964). Since the chains are covalently linked by disulfide bridges, they can be separated by reductive modification of the S–S bonds and subsequent gel filtration (Fleischman *et al.*, 1962). The isolated heavy chain retained varying amounts of the antigen binding capacity of the intact molecule and thus appeared essential for activity. The isolated light chain was devoid of immunologic activity (Fleischman *et al.*, 1963), but the question of whether it participates directly in the antibody-combining region has not yet been clarified. From equilibrium dialysis measurements Utsumi and Karush (1964) concluded that the active site of anti-phenyl β-lactoside antibody is exclusively associated with the heavy chain; they suggested that the light chain indirectly contributes to specificity by

* From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973. *Received June 2, 1965; revised October 29, 1965.* Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

† Present address: Virus Laboratory, University of California, Berkeley, California 94720.

combining with the heavy chain and preventing its denaturation and aggregation. On the other hand, Franèk and Nezlin (1963), Edelman *et al.* (1963), and Roholt *et al.* (1963b) found that the heavy chains from a variety of other antibodies possessed approximately 10% residual activity which was significantly enhanced by the subsequent addition of the homologous light chain. These results have been interpreted (Edelman *et al.*, 1963) to demonstrate a specific interaction of groups on both chains in the formation of the active site.

On the basis of these studies, the finding that the amino acid differences are all located in the light chains would throw considerable doubt on the relationship of the differences to antibody specificity. The finding that the differences are distributed in the heavy chain alone or in both the light and heavy chains would not only be consistent with the present information concerning the active site but would also help to elucidate the role of the light chain.

Methods

Preparation of Antisera. The immunizing antigens were synthesized by coupling at pH 9.0 (1) the diazonium salt of *p*-aminophenylarsonic acid to bovine γ-globulin, (2) the diazonium salt of *p*-aminophenyltrimethylammonium chloride to bovine serum albumin, and (3) the diazonium salt of *p*-aminophenyl β-lactoside to bovine serum albumin. The amounts of diazotized hapten added were twice the molar concentration of tyrosine present in the protein carrier. After dialysis to remove excess reagents, equal quantities of the bovine γ-globulin coupled to *p*-azophenylarsonic acid and the bovine serum albumin coupled to *p*-azophenyl β-lactoside were coprecipitated with alum. A second mixture was similarly prepared with equal quantities of bovine γ-globulin coupled to *p*-azophenylarsonic acid and bovine serum albumin coupled to *p*-azophenyltrimethylammonium chloride.

Each mixture was injected into New Zealand white rabbits in increasing dosage over a period of four weeks. The total dosage per animal was 120 mg of antigen. The animals were killed five days after the last injection. All the animals used were of the genotype $a^1a^1b^4b^4$. Their allotypic specificities were determined by the kind cooperation of Dr. Sheldon Dray at the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

The isolation of purified arsonic,[1] ammonium, and lac antibodies has been described fully (Koshland *et al.*, 1962, 1964, 1965). Briefly, the method consisted of the removal of antibody directed against the protein carrier by the addition of the appropriate antigen. The antihapten antibody was then precipitated with an azoantigen in which human fibrinogen was substituted as the protein carrier. Before the fibrinogen was coupled to neutral or positively charged diazonium salts, it was pretreated with iodoacetic acid to increase its net negative charge (Koshland *et al.*, 1965). The washed immune precipitates were dissolved in approximately 0.1 M solutions of the homologous hapten and the resulting solutions were applied to a diethylaminoethylcellulose column equilibrated with 0.02 M potassium phosphate buffer, pH 7.2. The negatively charged azofibrinogens were retained on the resin. The *p*-nitrophenylarsonate hapten used in the purification of arsonic antibody was also bound by the resin, and the antibody emerged as a single peak at column volume. In the purification of the other antibodies a mixture of the antibody and its respective hapten was eluted. The haptens were then separated by extensive dialysis against 0.01 M phosphate buffer 0.1 M in NaCl. When the efficiency of the separation methods was assayed by use of radioactive reagents (Koshland *et al.*, 1962, 1964), the amounts of antigen contaminants in the final product were found to be negligible. The moles of hapten which remained bound per mole of antibody ranged from less than 0.01 for arsonic antibody to 0.05 for ammonium antibody and 0.2–0.3 for lac antibody. However, the presence of these amounts of hapten contaminants was shown to have no detectable effect on the determinations of the amino acid content.

Reduction and Alkylation. The subunits of the arsonic, ammonium, and lac antibodies were obtained by the conventional method of reduction with mercaptoethanol, sometimes in the presence of urea, and subsequent alkylation of the free SH groups. The 2-mercaptoethanol (Eastman Organic Chemicals) was redistilled before use. The urea was purified by passing an 8 M solution through a mixed bed resin (Mallinckrodt Amberlite MB 3) and evaporating the eluate to dryness. Either of two alkylating agents was used; iodoacetamide (Mann Research Labs) which was twice recrystallized from the filtrate of a boiling water–charcoal mixture or iodoacetic acid (Eastman Organic Chemicals) which was recrystallized from warm petroleum ether until the product was white.

A range of reducing conditions was employed in the preparation of the light and heavy chains; the mercaptoethanol concentration was varied from 0.05 to 1.0 M and the urea from 0 to 12 M. When the mercaptoethanol alone was used, it was added to a 0.5–1.0% solution of the antibody in 0.1 M Tris buffer, pH 8.0. The solution was allowed to stand under nitrogen for 1 hr before an amount of dry iodoacetamide or iodoacetic acid in 10% molar excess of the mercaptoethanol concentration was added. The pH of the resulting solution was maintained between 7.5 and 8.0 for 0.5 hr with 5 N NaOH. When the reduction was carried out in the presence of urea, the reaction time was extended to 24 hr at room temperature so that maximum randomization of the protein structure would be achieved. The alkylated proteins were freed of the other products of the reaction by dialysis for 48 hr against five 4-l. changes of distilled water and then were taken to dryness in a rotary flash evaporator.

[1] The antibody to *p*-azophenylarsonic acid will be referred to subsequently as arsonic antibody; the antibody to *p*-azophenyltrimethylammonium ion as ammonium antibody; and the antibody to *p*-azophenyl β-lactoside as lac antibody.

Column Fractionation Procedures. The subunits of the three antibodies were separated by gel filtration through columns of Sephadex G-200 which was equilibrated with detergent to solubilize and dissociate the chains (Utsumi and Karush, 1964). The resin (Pharmacia, Upsala, Sweden) was prepared by washing six times with 0.01 M Tris buffer, pH 8.0, 0.001 M in Versene. After each addition of the buffer, the mixture was stirred for 0.5 hr and allowed to settle for 5–15 min before the supernatant fluid was removed. The resin was then washed four additional times with Tris–Versene buffer which contained 0.01 M sodium dodecyl sulfate or 0.05 M sodium decyl sulfate. The dodecyl sulfate (Fisher Scientific Co., USP) was used without further purification. The decyl sulfate was prepared by dissolving Dupanol 100 (E. I. du Pont Co.) in boiling absolute ethanol and allowing the detergent to crystallize from the cooled filtrate. Elemental analysis of the final product showed the values: H, 8.53; C, 46.74; S, 12.90; the theoretical values are 8.13, 46.13, and 12.32%, respectively.

The dried alkylated preparations were taken up in the Tris–detergent buffer to a concentration of 10–20 mg/ml. After standing for 4 hr at room temperature in the dodecyl sulfate buffer or overnight in the cold in the decyl sulfate buffer, any remaining insoluble material was removed by centrifugation. The clear supernatant contained at least 95% of the initial protein as determined by micro-Kjeldahl nitrogen analyses. Samples of less than 25 mg were fractionated on a column 1.4 × 100 cm; samples containing 25–125 mg were fractionated on a column 2.2 × 100 cm. The flow rate on the smaller column was 6–7 ml/hr and on the larger 12–15 ml/hr. The effluent was collected with a fraction collector in 2–2.5-ml fractions. The protein content of the individual fractions was monitored by readings in a Beckman spectrophotometer at 278 mμ. The resolution provided by the two different detergent buffer solutions was equivalent.

Amino Acid Analyses. Samples of the antibodies or their subunits were prepared for analysis by precipitation with sufficient 50% trichloroacetic acid so that its final concentration was 7–8%. The precipitates were washed with 3 ml of cold absolute ethanol and then air dried. Constant boiling HCl, 0.4 ml/mg of sample, was added and the contents were repeatedly frozen and thawed under vacuum to remove any dissolved oxygen (Crestfield *et al.*, 1963). The samples were subsequently hydrolyzed in a bath of constant boiling toluene at 110 ± 1° for 20 hr. The procedures of Spackman *et al.* (1958) were used in the analyses with the Beckman automatic analyzer. Equal quantities of each sample were applied to the short and long columns of the analyzer by use of the same pipet. The aliquot contained between 1 and 1.3 mg. A standard mixture of amino acids and an antibody hydrolysate were analyzed alternately and samples to be compared, *i.e.*, light chains from two antibodies prepared in the same rabbit, were analyzed with the same batch of ninhydrin solution.

The amino acid recoveries from intact antibody were normalized to a leucine content of 89, 89, and 91 residues, respectively, for the arsonic, lac, and ammonium antibodies. These values represent the average moles of leucine recovered per mole of antibody applied for nine preparations of each antibody. The moles of antibody applied were calculated from micro-Kjeldahl nitrogen analyses of the hydrolysates and the assumptions of a nitrogen content of 16% and a molecular weight of 160,000. The standard error of the mean in these values ranged from 0.7 to 1.1. In similar experiments the average number of leucines per 23,000 g of light chain was found to be 11.2 ± 0.07 for nine arsonic preparations, 11.3 ± 0.11 for five ammonium preparations, and 11.4 ± 0.39 for two lac preparations. The amino acid data for all three light chains were therefore normalized to a leucine content of 11. The leucine standards used for the heavy chains were derived by difference from the data on the whole molecules and their light chains.

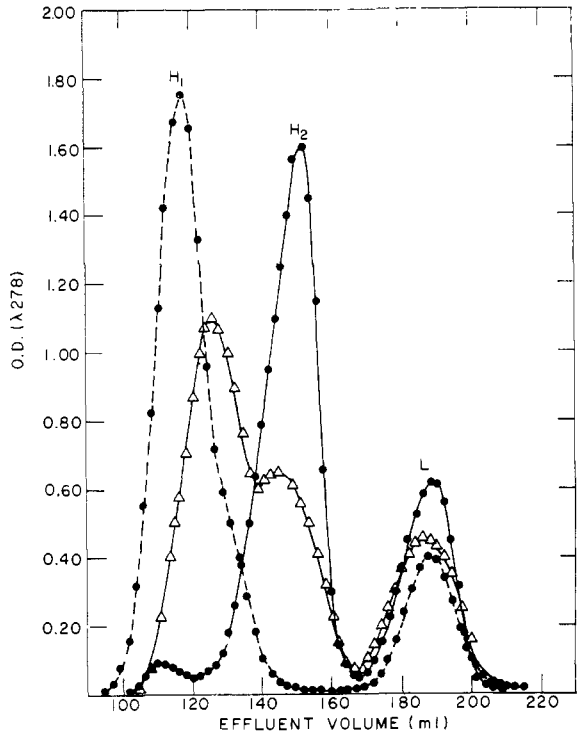

FIGURE 1: Fractionation of reduced and alkylated arsonic antibody from $a^1a^1b^4b^4$ double homozygous rabbits on Sephadex G-200. The broken line represents the separation after mild reduction with 0.05 M mercaptoethanol, the open triangles the separation after intermediate reduction with 0.1 M mercaptoethanol and 10 M urea, and the solid line the separation after complete reduction with 0.5 M mercaptoethanol and 12 M urea. Solvent: 0.01 M sodium dodecyl sulfate, 0.01 M Tris buffer, pH 8.0.

Results

Separation of Subunits by Gel Filtration. To determine

TABLE I: Effect of the Extent of Reduction on the Separation of Rabbit Antibody Subunits by Gel Filtration.

Antibody Preparation	No. of Expts	Recovered (%) Heavy Chain ($H_1 + H_2$)	Light Chain	Total
		After Mild Reduction		
Arsonic	5	74.5 ± 4.4	18.9 ± 1.7	93.4 ± 4.3
Ammonium	3	74.3 ± 3.7	18.5 ± 2.1	92.8 ± 2.9
		After Intermediate Reduction		
Arsonic	2	72.2 ± 8.5	22.6 ± 1.1	94.7 ± 9.5
Lac	3	71.2 ± 2.1	22.4 ± 2.3	93.6 ± 5.4
		After Complete Reduction		
Arsonic	6	68.3 ± 2.7	24.1 ± 1.2	92.4 ± 3.1
Ammonium	5	70.7 ± 1.1	25.2 ± 0.9	95.9 ± 1.5
Lac	3	65.7 ± 0.2	24.5 ± 0.3	90.2 ± 0.1

the optimum preparation of subunits for amino acid analysis, the three antibodies were treated with increasing amounts of reducing agent and the alkylated products were chromatographed on Sephadex G-200 equilibrated with detergent. Representative examples of the separations achieved are given in Figure. 1. After mild reduction, all the heavy chain was recovered in the fastest moving H_1 fraction with only a shoulder on the descending limb to indicate the presence of small amounts of slower moving material. With intermediate conditions of reduction the heavy chain was distributed between the H_1 fraction and the second, slower moving H_2 fraction, while after complete reduction the heavy chain was eluted almost exclusively in the H_2 fraction. In contrast, the filtration rate of the third component, the light chain, was independent of the extent of disulfide bond cleavage. These patterns were in good agreement with those previously reported by Utsumi and Karush (1964) from similar gel filtration studies.

The yields obtained by the three representative methods of preparation are summarized in Table I. The amounts present in each fraction were determined from the readings at 278 mμ without correction for differences in the absorbancies among the intact molecule and its chains. The standard errors of an individual determination were included to show the reproducibility of the data. It may be seen that with more stringent reducing conditions there was a significant increase in the per cent recovered in the light chain fraction and a corresponding decrease in the per cent recovered in the heavy chain fractions. The lower yields of light chain after mild reduction could not be explained by losses during chromatography since the elution of protein was essentially quantitative in all experiments and since at each level of reduction the yields of light and heavy chains could be reproduced within at least 10%. The lower yields also could not be attributed to an inhibitory effect of hapten; the same yields were observed for the three antibodies although the residual hapten varied on a molar basis from less than 0.1% in the arsonic antibody preparations to 5% in the ammonium antibody and 20–30% in the lac antibody preparations.

The data from the arsonic antibody fractionations were corrected for differences in absorbancies by measurements of the extinction coefficients in 0.01 M phosphate buffer 0.05 M in decyl sulfate and pH 8.0. The values found with protein solutions of known nitrogen content were 14.5 for the intact antibody and 12.8 for the light chain. On this basis, the corrected recoveries of arsonic light chain ranged from 20.8% after mild reduction to 27.3% after complete reduction. These results are in conflict with previous work in which maximum yields of 28% were obtained even after mild reduction (Utsumi and Karush, 1964).

Amino Acid Compositions of the Heavy Chains from Purified Rabbit Antibodies. To clarify the results of the gel filtration experiments, amino acid analyses were carried out on the various preparations of partially and fully reduced heavy chains. Representative data obtained with the heavy chains of arsonic antibody after 20 hr of acid hydrolysis are given in Table II. The H_1 fraction from mild reduction was found to have a significantly different amino acid composition than the H_2 fraction from complete reduction (*cf.* values in column 1 with those in column 5). Its carboxymethylcysteine content was 3.3 residues per mole, more than the yield of 2 required by the cleavage of the disulfide bridge linking the light chain and the disulfide bridge linking the other heavy chain. However, it was possible that the reduction of the interchain bonds did not exclusively precede the reaction of intrachain disulfides and thus the differences in amino acid composition were due to the presence of residual light chain. To test this possibility, the amino acid composition of a theoretical mixture of 90% fully reduced heavy chain and 10% light chain was calculated. The values are listed in column 2 of the table and their excellent agreement with the experimentally determined composition of the mildly reduced heavy chain demonstrated that the separation of the subunits was not complete under

TABLE II: Average Amino Acid Recoveries[a] for Heavy Chain Preparations of Arsonic Antibody.

Extent of Reduction	Mild		Intermediate		Complete
Gel Filtration Fraction	H_1[b]	Theoretical 90% heavy 10% light	H_1[c]	H_2[c]	H_2[d]
Amino Acid	Residues per 53,000 gm				
Lys	25.3	25.7	25.2	25.4	25.3
His	6.50	6.43	6.78	6.79	6.72
Arg	18.4	18.7	19.4	19.2	19.7
Asp	34.9	35.3	34.0	33.5	33.1
Thr	53.4	54.5	50.8	50.2	50.4
Ser	53.8	54.3	52.6	52.8	53.1
Glu	41.2	41.8	39.7	39.5	39.3
Pro	42.7	42.0	43.2	43.0	42.9
Gly	36.7	37.1	35.0	35.0	34.6
Ala	25.0	25.6	24.1	23.4	23.0
Val	44.6	44.4	42.8	42.4	42.4
Met	5.73	5.67	5.85	6.17	5.97
Ileu	17.2	17.0	16.6	16.6	16.4
Leu	33	33	33	33	33
Tyr	18.7	18.6	17.5	17.3	17.1
Phe	15.6	15.7	15.5	15.5	15.3
CM-Cys	3.30		8.73	11.3	13.7

[a] Hydrolysis time, 20 hr. [b] Average values from three different preparations. [c] One preparation. [d] Average values from six different preparations.

these conditions of reduction. Furthermore, the finding that each heavy chain was contaminated with approximately 10% light chain correlated well with the lower yields, 75–80% of the maximum, observed for the corresponding light chains on gel filtration.

Amino acid recoveries similar to those of the fully reduced heavy chain were obtained only with preparations in which at least ten cysteines had been alkylated. It would appear, therefore, that the reduction of 4–5 intrachain disulfide bonds proceeds at a rate comparable to their interchain bonds. Thus their cleavage is also required in order to isolate pure heavy chain. As an example, the analyses of heavy chain prepared with intermediate conditions of reduction, *i.e.*, 0.1 M mercaptoethanol and 10 M urea, are shown in columns 3 and 4 of Table II. The H_1 fraction had a carboxymethylcysteine recovery of 8.7 residues per mole. Although not statistically significant, there was an indication of light chain contamination in the higher aspartic acid and alanine yields. The H_2 fraction, on the other hand, had a carboxymethylcysteine recovery of 11.3 per mole and its amino acid composition was identical with that of the fully reduced heavy chain within the limits of detection. Since only trace amounts of light chain were found in the H_1 component, the two gel filtration fractions of heavy chain could not be explained simply as a complex of light and heavy chain *vs.* pure heavy chain. The differences in the rate of filtration would appear to reflect other properties such as the aggregation or swelling of the partially reduced chain.

When preparations of partially and fully reduced heavy chains from ammonium antibody were assayed for their amino acid content, data comparable to those presented above for arsonic antibody subunits were obtained. Thus the required cleavage of at least six disulfide bonds for the isolation of pure heavy chain was not due to the special structure of one antibody. On the basis of these studies, fully reduced samples were chosen as the optimum material for the determinations of the amino acid composition of the heavy chains. The average values from the analyses of six preparations of arsonic heavy chains, six of ammonium heavy chains, and three of lac heavy chains after reduction with 1 M mercaptoethanol and 12 M urea are given in Table III. The accompanying standard errors of the mean showed that the yields were very reproducible. No individual result differed by more than 3% from the average except in the determinations of the methionine and histidine contents, and in these the maximum deviation from the mean was 6%.

The amino acid compositions of the three heavy chains were for the most part strikingly similar. However, a few small differences were noted. In comparison to the arsonic heavy chain, the ammonium heavy chain

TABLE III: Average Amino Acid Recoveries[a] from the Heavy Chains of Three Antibodies Isolated from $a^1a^1b^4b^4$ Rabbits.

Amino Acid	Residues per 53,000 g		
	Arsonic Antibody	Ammonium Antibody	Lac Antibody
Lys	25.3 ± 0.12[b]	25.4 ± 0.17[b]	25.5 ± 0.098[a]
His	6.73 ± 0.039	6.87 ± 0.13	7.17 ± 0.11
Arg	19.7 ± 0.089	19.5 ± 0.16	19.6 ± 0.058
Asp	33.1 ± 0.25	34.5 ± 0.19	33.5 ± 0.30
Thr	50.4 ± 0.31	50.7 ± 0.28	49.9 ± 0.098
Ser	53.1 ± 0.25	52.4 ± 0.27	50.3 ± 0.36
Glu	39.3 ± 0.12	40.0 ± 0.13	39.2 ± 0.32
Pro	42.9 ± 0.25	43.1 ± 0.29	41.9 ± 0.26
Gly	34.6 ± 0.18	34.9 ± 0.049	34.5 ± 0.36
Ala	23.0 ± 0.29	23.1 ± 0.13	22.7 ± 0.36
Val	42.4 ± 0.24	42.5 ± 0.20	42.0 ± 0.31
Met	5.97 ± 0.078	5.93 ± 0.16	6.11 ± 0.081
Ileu	16.4 ± 0.078	16.3 ± 0.065	16.2 ± 0.13
Leu	33	34	33
Tyr	17.1 ± 0.13	17.0 ± 0.12	14.9 ± 0.042
Phe	15.3 ± 0.057	15.3 ± 0.053	15.1 ± 0.12
CM-Cys	13.7 ± 0.058	13.8 ± 0.18	13.2 ± 0.13

[a] Hydrolysis time, 20 hr. [b] Standard error of the mean.

contained an additional residue of aspartic acid and an additional residue of leucine. By the application of the Student t test, the differences in the aspartic acid recoveries were shown to be significant at the 99.9% confidence level and, although statistical analysis could not be applied to the leucine values because of the normalization procedures, the differences in content were buttressed by the excellent agreement in the yields of all the remaining amino acids except aspartic acid. The extra leucine per mole of ammonium heavy chain accounted for the recoveries of 89 *vs.* 91 leucines in the comparative analyses of the intact arsonic and ammonium antibodies. The increased content of aspartic acid in the ammonium heavy chain, however, accounted for only two of the four residue difference observed in the whole molecules.

Similar statistical analyses showed that the yields of two amino acids in the lac heavy chain differed at the 99.9% confidence level from those of either arsonic or ammonium heavy chain. The lac heavy chain contained three fewer serine residues and two fewer tyrosine residues. These results indicated that all of the serine differences and four of the six tyrosine residue differences noted in the amino acid composition of the intact lac antibody were located in its heavy chain. In addition, evidence was obtained for a new difference in the proline content of the lac heavy chain since the probability that the lac heavy chain had one less proline was found to be 0.95.

Amino Acid Compositions of the Light Chains from Purified Rabbit Antibodies. The effect of the extent of reduction on the separation of light chain was also determined. The data from the light chains of arsonic antibody isolated after mild reduction with 0.05 M mercaptoethanol and after extensive reduction with 0.5 M mercaptoethanol and 12 M urea are presented in Table IV. It may be seen that the amino acid recoveries from the mildly reduced preparation (column 1) were identical with those of the completely reduced preparations (column 4) except for the carboxymethylcysteine yields. Furthermore, the recovery of one carboxymethylcysteine residue per mole of the mildly reduced light chain indicated that the disulfide bond linking the light chain to the heavy chain was more readily reduced than any of the three intra(light chain) disulfide bonds.

The purity of the mildly reduced light chain was ascertained by passing the preparation through Dowex 1 resin in the acetate form to remove the detergent (Cebra, 1964). The preparation was then resolubilized in detergent and rechromatographed on Sephadex G-200 under the conditions used in the initial separation. No evidence of contamination with heavy chain was found in the rerun of the light chain component shown in Figure 2. This result was supported by the analyses of the mildly reduced light chain after the removal of the detergent and after rechromatography (data in columns 2 and 3, respectively, of Table IV). There were no significant changes in the amino acid yields compared to those of the original gel filtration fraction (column 1). When these experiments were repeated with ammonium antibody light chain, its amino acid composition was also found to be independent of the

TABLE IV: Average Amino Acid Recoveries[a] for Light Chain Preparations of Arsonic Antibody.

Extent of Reduction	Mild			Complete
Gel Filtration Fraction	Untreated[b]	Detergent[b] Removed	Rerun on[b] G-200	Untreated[c]
Amino Acid	Residues per 23,000 g			
Lys	9.26	9.21		9.20
His	1.29	1.24		1.33
Arg	3.20	3.22		3.00
Asp	19.0	19.0	19.0	19.3
Thr	30.6	30.4	30.6	30.5
Ser	21.9	21.8	21.8	21.3
Glu	21.6	21.4	21.3	21.4
Pro	12.1	12.1	11.9	12.0
Gly	19.6	19.6	19.7	19.9
Ala	16.4	16.3	16.3	16.6
Val	20.0	20.2	21.0	20.9
Met	0.91	0.92	0.90	0.87
Ileu	7.39	7.40	7.41	7.56
Leu	11	11	11	11
Tyr	10.8	10.4	10.6	10.9
Phe	6.50	6.45	6.60	6.57
CM-Cys	0.95	0.96	0.95	7.13

[a] Hydrolysis time, 20 hr. [b] Values represent one analysis. [c] Values represent average from two different preparations.

conditions of reduction and the yields of light chain obtained on gel filtration.

The amino acid compositions of the light chains given in Table V were determined, therefore, from the analyses of both mildly reduced and extensively reduced samples. The values for the arsonic light chain represent the average of eight different preparations, the values for the ammonium light chain the average of five different preparations, and the values of the lac light chain the average of four different preparations. The assays of each antibody chain were remarkably reproducible; excluding the methionine recoveries the average per cent errors calculated from the standard errors of the mean listed in Table IV were 0.69, 0.58, and 1.1 for the arsonic, ammonium, and lac data, respectively.

Although the compositions of the three light chains were generally similar, the application of the t test revealed a number of differences which were significant at the 99.9% confidence level. Approximately one-half of these were differences of a whole residue or multiples thereof; the remaining was differences of fraction of residues. Part of these results was due to the location in the light chains of differences previously observed in the amino acid compositions of the intact molecules. In the case of the ammonium light chain, the smaller yields of arginine and isoleucine were consistent with the lower content of these residues in the intact antibody. Furthermore, the ammonium light chain contained one more aspartic acid residue than the corresponding light chain of arsonic antibody. This extra aspartic acid explained two of the four residue differences in the total aspartic acid content of arsonic and ammonium antibodies. The other two were already accounted for by the extra aspartic acid which appeared in the ammonium heavy chain. In the case of the lac

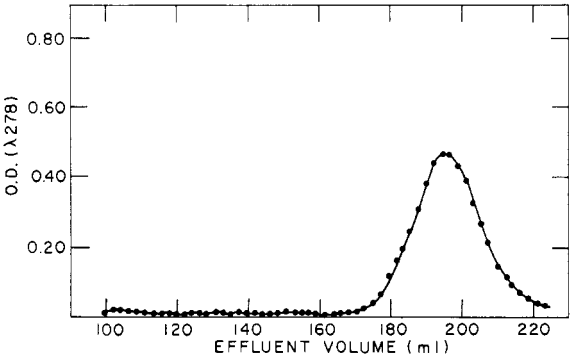

FIGURE 2: Rerun on Sephadex G-200 of the separated light chain from mildly reduced and alkylated arsonic antibody. Solvent: 0.01 M sodium dodecyl sulfate, 0.01 M Tris buffer, pH 8.0.

TABLE V: Average Amino Acid Recoveries[a] from the Light Chains of Three Antibodies Isolated from $a^1a^1b^4b^4$ Rabbits.

Amino Acid	Residues per 23,000 g		
	Arsonic Antibody	Ammonium Antibody	Lac Antibody
Lys	9.27 ± 0.081[b]	9.20 ± 0.036[a]	9.81 ± 0.037[a]
His	1.27 ± 0.028	1.27 ± 0.014	1.40 ± 0.022
Arg	3.05 ± 0.035	2.61 ± 0.060	3.20 ± 0.056
Asp	19.1 ± 0.12	20.2 ± 0.058	21.8 ± 0.17
Thr	30.5 ± 0.11	30.2 ± 0.043	31.0 ± 0.16
Ser	21.6 ± 0.12	21.4 ± 0.085	21.5 ± 0.12
Glu	21.2 ± 0.12	20.8 ± 0.063	20.7 ± 0.18
Pro	11.9 ± 0.046	12.1 ± 0.043	12.7 ± 0.07
Gly	19.9 ± 0.078	19.9 ± 0.098	19.9 ± 0.10
Ala	16.5 ± 0.10	15.5 ± 0.014	15.8 ± 0.22
Val	20.8 ± 0.14	21.7 ± 0.15	22.5 ± 0.12
Met	0.87 ± 0.011	0.41 ± 0.020	0.86 ± 0.046
Ileu	7.47 ± 0.029	6.79 ± 0.030	7.31 ± 0.080
Leu	11	11	11
Tyr	10.8 ± 0.11	10.9 ± 0.067	10.1 ± 0.14
Phe	6.57 ± 0.024	6.77 ± 0.030	7.06 ± 0.060
CM-Cys[c]	7.26 ± 0.021	7.08 ± 0.054	7.03 ± 0.25

[a] Hydrolysis time, 20 hr. [b] Standard error of the mean. [c] Average of three different determinations.

light chain there were three additional aspartic acid residues and one less tyrosine residue compared to the arsonic light chain. Thus all of the aspartic acid and part of the tyrosine differences between arsonic and lac antibodies were found to be located in their respective light chains.

In addition, new differences appeared in the analyses of the light chains which had not been recognized in the previous analyses of the whole antibodies. In some cases the differences were not seen because of a compensating change in the content of the homologous heavy chain. For example, although the light chain of lac antibody had an additional residue of proline compared to the arsonic or ammonium light chain, its heavy chain was shown to have one less. Thus the total proline recovery was identical with those of the other two antibodies. In other cases the differences may have been obscured by the mixed allotypy of the samples. For example, the data in Table V show that the light chain of arsonic antibody contained one more alanine residue, *i.e.*, 16.5 residues *vs.* 15.5 for the ammonium light chain and 15.8 for the lac light chain, and one or two less valine residues, *i.e.*, 20.8 *vs.* 21.7 for the ammonium light chain and 22.5 for the lac light chain. These differences were found to be reflected in the total yields when the analyses were carried out on the antibodies of allotypic specificity a^1b^4 from which the chains were derived (Tables VI, VII, and VIII). The alanine values were 80.3, 79.1, and 77.4 and the valine values were 128, 130, and 131 for arsonic, ammonium, and lac antibodies, respectively.

Finally, many of the amino acid yields in the three light chains were consistently fractions of residues and differed significantly from each other by fractions of residues. This was particularly apparent in the histidine recoveries of 1.27, 1.27, and 1.40 and the methionine recoveries of 0.87, 0.41, and 0.86 per mole of the respective light chains. The deviations from whole numbers in these residues could not be satisfactorily explained on the basis of impurities because the results were reproducible even after rechromatography and removal of the detergent from the preparations. They also could not be explained by errors in the molecular weight determinations since the magnitude required would be a factor of two. The finding of fractional recoveries was interpreted, therefore, to show that the light chain of each antibody was not a single homogeneous entity but was composed of at least two chains which differed slightly from each other in their amino acid content. Furthermore, the reproducibility of the fractional yields indicated that the combination of light chains in each antibody remained constant from one preparation to another. These results were in agreement with the recurring heterogeneity observed by Cohen (1963) and Cohen and Porter (1964); they found that the light chains from immunoglobulins of all species examined had at least ten well-separated components on gel electrophoresis.

In addition to a multiplicity of structure, the three light chains also exhibited consistent differences in their average amino acid compositions. Thus, the combination of light chains in each of the rabbit antibodies studied appeared to be specific for that antibody. Evidence for such a specific combination of light chains

has been obtained with antibodies from other species (guinea pig antibodies, Edelman *et al.*, 1961, and mouse antihapten antibodies, Merryman and Benacerraf, 1963). In these studies the light chains of each antibody were found to give characteristic band patterns under restricted conditions of gel electrophoresis.

Correlation of the Amino Acid Compositions of Rabbit Antibodies and Their Subunits. Amino acid analyses were carried out on samples of the intact arsonic, ammonium, and lac antibodies from which the chains were prepared. The purpose of these experiments was twofold: (1) to determine the amino acid compositions of the three antibodies when they were isolated from rabbits homozygous for γ-globulin production and (2) to correlate the data from the subunits with those of the intact antibody. The amino acid composition of arsonic antibody shown in Table VI is the average yield from seven different preparations after 20 hr of acid hydrolysis. The data for ammonium antibody (Table VII) is the average of six preparations and the data for lac antibody is the average of eight (Table VIII).

The three amino acid compositions differed only in minor respects from those previously reported for antibodies of unknown allotypic specificities (Koshland and Englberger, 1963; Koshland *et al.*, 1964). Some of the variation, *i.e.*, in the glutamic acid and serine yields, was found to be due to changes in the contents of these residues in the standard amino acid mixture. The remaining differences were surprisingly few, *i.e.*, in the alanine and valine yields, especially since Reisfeld *et al.* (1965) recently reported large differences in the amino acid compositions of light chains prepared from the γ-immunoglobulins of b^4 and b^5 homozygous rabbits. Their differences amounted to 15 residues and involved seven or eight amino acids.

In view of these results, the allotypic specificities of many of the previous preparations of purified antibodies were determined.[2] The preparations were found to exhibit only b^4 specificity at the b locus on the light chain and primarily a^1 or both a^1 and a^3 at the a locus on the heavy chain. Thus, by chance, the antibodies previously studied were essentially representative of homozygous a^1b^4 γ-globulin production. On the basis of these genetic analyses, the striking similarity between the earlier amino acid compositions and those presented here would be expected.

The composition of the intact antibodies was also calculated from the amino acids present in their respective light and heavy chains and from the assumption of two pairs of each chain per antibody molecule. The calculated values given in column 3 of Tables VI, VII, and VIII agreed with the experimentally determined values for each antibody within the error of the analyses. The excellent correlations obtained not only supported the working model of a four-chain structure, but also provided additional evidence that the chain preparations were pure and, therefore, the measurements of their amino acid compositions were meaningful.

TABLE VI: Correlation of the Amino Acid Recoveries[a] of Arsonic Antibody and Its Subunits Prepared from $a^1a^1b^4b^4$ Rabbits.

Amino Acid	Residues per mole[b]			
	Heavy Chain	Light Chain	$2\left(\frac{\text{Heavy}}{+\text{ Light}}\right)$	Intact Antibody
Lys	25.3	9.3	68	70.3 ± 0.25[c]
His	6.7	1.3	16	16.5 ± 0.14
Arg	19.7	3.1	46	45.0 ± 0.19
Asp	33.1	19.1	104	105 ± 0.53
Thr	50.4	30.5	162	161 ± 1.10
Ser	53.1	21.6	149	149 ± 0.57
Glu	39.3	21.2	120	122 ± 0.28
Pro	42.9	11.9	110	110 ± 0.87
Gly	34.6	19.9	110	110 ± 0.64
Ala	23.0	16.5	80	80.2 ± 0.48
Val	42.4	20.8	127	128 ± 0.72
Met	6.0	0.9	14	13.6 ± 0.16
Ileu	16.4	7.5	48	48.7 ± 0.36
Leu	33	11	88	89
Tyr	17.1	10.8	56	56.8 ± 0.57
Phe	15.3	6.6	44	44.1 ± 0.16
CM-Cys	13.7	7.3	42	42.5[d]

[a] Hydrolysis time, 20 hr. [b] The molecular weights of the heavy chain, light chain, and whole antibody were assumed to be 53,000, 23,000, and 160,000 respectively. [c] Standard error of the mean. [d] One determination.

Discussion

The data presented here demonstrate that the differences previously observed in the amino acid compositions of three rabbit antibodies are located in both their heavy and light chains. Thus, not only does each antibody have a characteristic amino acid composition, but also its heavy and its light chains have characteristic amino acid compositions. These data are consistent with the findings of others that both chains contribute directly or indirectly to the specificity of binding (Franèk and Nezlin, 1963; Edelman *et al.*, 1963; Utsumi and Karush, 1964; Roholt *et al.*, 1963b). The correlation provides further evidence that the observed amino acid differences are associated with immunological reactivity and represent changes in the contact amino acids at the active site or in residues which determine the three-dimensional arrangement of the site. This conclusion is also supported by recent studies (Haber, 1964; Whitney and Tanford, 1965) in which an active antibody fragment consisting of one light chain and the N-terminal half of a heavy chain was completely reduced and unfolded and then allowed to reoxidize spontaneously. Since the amount of activity recovered was at

[2] We are indebted to Mr. Leroy Gerald, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, for the analyses of the allotypic specificities.

TABLE VII: Correlation of the Amino Acid Recoveries[a] of Ammonium Antibody and Its Subunits Prepared from $a^1a^1b^4b^4$ Rabbits.

Amino Acid	Residues per mole[b]			
	Heavy Chain	Light Chain	2(Heavy + light)	Intact Antibody
Lys	25.4	9.2	68	69.7 ± 0.35[c]
His	6.9	1.3	16	16.7 ± 0.12
Arg	19.5	2.6	44	43.3 ± 0.19
Asp	34.5	20.2	110	110 ± 0.61
Thr	50.7	30.2	162	162 ± 1.1
Ser	52.4	21.4	148	148 ± 0.78
Glu	40.0	20.8	122	122 ± 0.69
Pro	43.1	12.1	110	111 ± 0.86
Gly	34.9	19.9	110	110 ± 0.25
Ala	23.1	15.5	78	79.1 ± 0.45
Val	42.5	21.7	130	130 ± 0.45
Met	5.9	0.4	13	12.9 ± 0.17
Ileu	16.3	6.8	46	46.6 ± 0.35
Leu	34	11	90	91
Tyr	17.0	10.9	56	56.2 ± 0.45
Phe	15.3	6.8	44	44.4 ± 0.24
CM-Cys	13.8	7.1	42	

[a] Hydrolysis time, 20 hr. [b] The molecular weights of the heavy chain, light chain, and intact antibody were assumed to be 53,000, 23,000, and 160,000 respectively. [c] Standard error of the mean.

TABLE VIII: Correlation of the Amino Acid Recoveries[a] of Lac Antibody and Its Subunits Prepared from $a^1a^1b^4b^4$ Rabbits.

Amino Acid	Residues per mole[b]			
	Heavy Chain	Light Chain	2(Heavy + light)	Intact Antibody
Lys	25.5	9.8	71	70.3 ± 0.34[c]
His	1.4	7.2	17	17.1 ± 0.081
Arg	19.6	3.2	46	45.2 ± 0.24
Asp	33.5	21.8	112	111 ± 0.50
Thr	49.9	31.0	162	162 ± 0.79
Ser	50.3	21.5	144	143 ± 0.48
Glu	39.2	20.7	120	120 ± 0.50
Pro	41.9	12.7	110	110 ± 0.29
Gly	34.5	19.9	110	109 ± 0.76
Ala	22.7	15.8	78	77.4 ± 0.49
Val	42.0	22.5	130	131 ± 0.81
Met	6.1	0.9	14	13.5 ± 0.19
Ileu	16.2	7.3	47	47.6 ± 0.33
Leu	33	11	88	89
Tyr	14.9	10.1	50	50.5 ± 0.13
Phe	15.1	7.1	44	44.5 ± 0.13
CM-Cys	13.2	7.0	40	

[a] Hydrolysis time, 20 hr. [b] The molecular weights of heavy chain, light chain, and the intact antibody were assumed to be 53,000, 23,000, and 160,000, respectively. [c] Standard error of the mean.

least 100 times that expected by random re-formation of the disulfide bonds, the antibody specificity appeared to be determined by the primary sequence of amino acids.

The analyses suggest that the heavy chains of rabbit antibodies are homogeneous with respect to their amino acid composition. Although only two of the amino acids, histidine and methionine, are present in sufficiently small amounts to be useful as evidence for multiple structures, in both these instances the average yields are essentially whole residues, 7 moles of histidine and 6 moles of methionine for each antibody heavy chain. These limited data are in agreement with other studies of the homogeneity of rabbit heavy chains; for example, the number of peptides obtained after trypsin digestion corresponds to the number calculated for a homogeneous chain, *i.e.*, the number of lysine and arginine residues per molecule (Small *et al.*, 1965).

In contrast, the analyses show that the light chains of each antibody are heterogeneous with respect to their amino acid composition. The most striking example is the methionine content of the ammonium light chain. The recovery of 0.41 mole indicates that more than one-half the light chains present contain no methionine. Significant heterogeneity is also apparent in the lysine, histidine, arginine, isoleucine, and phenylalanine yields in the three light chains studied. These data offer a chemical explanation for some of the light chain complexity observed in many previous studies of their biological properties, electrophoretic mobilities, etc. Since the yields of three amino acids, glutamine, asparagine, and tryptophan, were not determined in these studies, some of the heterogeneity could also result from variations in their content. However, these data do not resolve the biological origin of the light chain complexity. The amino acid compositions were determined on samples isolated from b^4 homozygous rabbits so that the heterogeneity cannot be attributed to known genetic types of γ-globulin. It may reflect unknown allotypic determinants or other variables, such as production by different cell types.

Despite the multiplicity of chains involved, the average amino acid composition of the light chain preparation from each antibody is specific for that antibody. Thus, the amino acid composition of the light chain of lac antibody or ammonium antibody is reproducible and distinguishable from the light chain of arsonic antibody. Moreover, in some cases the differences in the light chains can by themselves account for the differences obtained in the whole antibodies. For example, the aspartic acid content of lac and arsonic light chains differs by three residues and therefore these values satisfactorily account for the difference of six in the aspartic acid recoveries of the whole antibodies.

It is interesting that most of the charged residue differences among arsonic, ammonium, and lac antibodies are located in their light chains. This distribution pattern may explain the characteristic bands obtained for guinea pig and mouse antihapten antibodies after electrophoresis at pH 3.5 (Edelman *et al.*, 1961; Merryman and Benacerraf, 1963) and the differences in the relative staining intensities of the bands following electrophoresis at neutral pH (Cohen, 1963).

The finding that the light chain of each antibody has a characteristic average amino acid composition does not delineate its precise role in specificity. Side chains from the light chain may participate directly in the binding of hapten at the active site as the labeling experiments of Metzger *et al.* (1963) and Roholt *et al.* (1963a) suggest. On the other hand, residues in the light chain may act at a distance by stabilizing the conformation of binding groups in the heavy chain as Utsumi and Karush (1964) suggest. Furthermore, the contribution of the light chain may vary from one antibody to another. It is evident from this discussion that more detailed studies of antibody light chains, such as the use of reporter groups (Burr and Koshland, 1964), the modification of specific residues, etc., are needed before this question can be resolved.

One hypothesis to explain the finding of a characteristic average amino acid composition on the one hand and of multiple structures on the other is that the light chains of each antibody contain two regions identified with specificity. One of these is "invariant" in amino acid composition[3] in all light chains present for a given antibody. It provides the major contribution of the light chain to the specificity of the antibody. The second of these is "variable" in amino acid composition and serves to modify the quantitative effect of the "invariant" region. This mechanism would explain both the specificity of the homologous light chains in recombination experiments and some of the heterogeneity of binding at the active site. Moreover, the changes in affinities observed by Eisen and Siskind (1964) might on this hypothesis be explained by an increased production of one form of light chain at the expense of another. If the latter explanation is correct, it would be predicted that the average amino acid composition of the light chain, except for the invariant region, would change as a function of the length of immunization.

[3] This region might be a limited consecutive sequence of amino acids or one or more residues always appearing in approximately the same three-dimensional arrangement.

References

Burr, M., and Koshland, D. E., Jr. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1017.

Cebra, J. J. (1964), *J. Immunol. 92*, 977.

Cohen, S. (1963), *Biochem. J. 88*, 2p.

Cohen, S., and Porter, R. R. (1964), *Biochem. J. 90*, 278.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Edelman, G. M. (1959), *J. Am. Chem. Soc. 81*, 3155.

Edelman, G. M., Benacerraf, B., Ovary, A., and Poulik, M. D. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1751.

Edelman, G. M., Olins, D. E., Gally, J. A., and Zinder, N. D. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 753.

Edelman, G. M., and Poulik, M. D. (1961), *J. Exptl. Med. 113*, 861.

Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.

Fleischman, J. B., Pain, R. H., and Porter, R. R. (1962), *Arch. Biochem. Biophys. Suppl. 1*, 174.

Fleischman, J. B., Porter, R. R., and Press, E. M. (1963), *Biochem. J. 88*, 220.

Franèk, F., and Nezlin, R. S. (1963), *Folia Microbiol. Prague 8*, 128.

Haber, E. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1099.

Koshland, M. E., and Englberger, F. M. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 61.

Koshland, M. E., Englberger, F. M., and Gaddone, S. M. (1962), *J. Immunol. 89*, 517.

Koshland, M. E., Englberger, F. M., and Gaddone, S. M. (1965), *Immunochemistry 2*, 115.

Koshland, M. E., Englberger, F. M., and Shapanka, R. (1964), *Science 143*, 1330.

Marler, E., Nelson, C. A., and Tanford, C. (1964), *Biochemistry 3*, 279.

Merryman, C., and Benacerraf, B. (1963), *Proc. Soc. Exptl. Biol. Med. 114*, 372.

Metzger, H., Wofsey, L., and Singer, S. J. (1963), *Biochemistry 2*, 979.

Pain, R. H. (1963), *Biochem. J. 88*, 234.

Porter, R. R. (1962), *in* The Symposium on Basic Problems of Neoplastic Disease, Gellhorn, A., and Hirschberg, E., Ed., New York, N. Y., Columbia University Press, p 177.

Reisfeld, R. A., Dray, S., and Nisonoff, A. (1965), *Immunochemistry 2*, 155.

Roholt, O., Onoue, K., and Pressman, D. (1963a), *Proc. Natl. Acad. Sci. U. S. 51*, 173.

Roholt, O., Radzimski, G., and Pressman, D. (1963b), *Science 141*, 726.

Small, P. A., Jr., Kehn, J. E., and Lamm, M. E. (1963), *Science 142*, 393.

Small, P. A., Jr., Reisfeld, R. A., and Dray, S. (1965), *Federation Proc. 24*, 201.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Utsumi, S., and Karush, F. (1964), *Biochemistry 3*, 1329.

Whitney, P. L., and Tanford, C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 524.