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*DIFFERENCES IN THE AMINO ACID COMPOSITION OF TWO PURIFIED ANTIBODIES FROM THE SAME RABBIT**

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In previous studies[1,2] no significant differences could be demonstrated in the amino acid content of rabbit antibodies of different specificity. These data have been used to support the theories of antibody formation[3, 4] which postulate that specificity results from a three-dimensional rearrangement of peptide chains in γ globulin. However, a significant change in the steric arrangement of the active site might be caused by a relatively small percentage change in the total number of amino acids. Such a change could have been obscured by the experimental error in the analytical procedures used. For example, with the exception of methionine, histidine, and tryptophan, more than 40 moles of each amino acid have been recovered per mole of antibody. A change of two residues, one at each active site, would result therefore in a maximum difference of 5 per cent in the total content of these amino acids.

Recently, the experimental error in such measurements has been considerably reduced by the development of (*a*) the automatic amino acid analyzer[5] and (*b*) improved methods for the isolation of antihapten antibodies[6, 7] whose purity can be assayed by as stringent criteria as equilibrium dialysis.[8, 9] The purpose of the present research was to re-examine the amino acid composition of two rabbit antibodies with the improved analytic accuracy of these methods. Antibodies of completely unrelated specificity, one directed against the negatively charged *p*-azobenzenearsonic acid group and the other against the positively charged *p*-azophenyltrimethylammonium ion, were chosen to provide the optimum material for the detection of differences in the amino acid content. Furthermore, to minimize the possibility that the results might reflect genetic differences in the pools from which the antibodies were obtained, experiments were performed in which the two antibodies were isolated from the serum of single rabbits.

Preparation of antisera: The two immunizing antigens were synthesized by coupling (1) 10 micromoles of bovine γ globulin to 1.2 millimoles of diazotized arsanilic acid, and (2) 10 micromoles of bovine serum albumin to 0.4 millimole of the diazonium salt of *p*-aminophenyltrimethylammonium chloride. After exhaustive dialysis to remove excess reagent, the azoproteins were precipitated with alum by the method of Karush and Marks.[6] Antisera were prepared in New Zealand white rabbits which were injected with increasing amounts of antigen over a period of four weeks and exsanguinated five days after the last inoculation. Each animal received a total of 80 mg, composed either of a single antigen or of equal amounts of both antigens.

Purification of antibody: The method employed for the isolation of arsonic antibody[10] has been described in detail elsewhere.[7] Briefly, it consists of the preliminary removal of antiprotein carrier antibody and complement by the addition of 14 μg of bovine γ globulin N/ml of antisera. The antihapten antibody was then precipitated with 12–16 μg N/ml of an azoantigen in which human

fibrinogen was substituted as the protein carrier. The washed immune precipitate was dissolved in 0.025 *M* *p*-nitrobenzenearsonic acid, and the resulting solution was applied to a DEAE-cellulose column equilibrated with 0.02 *M* phosphate buffer, pH 7.2. The antibody appeared in the eluate as a single peak at column volume.

A similar procedure was used to purify the anti-*p*-azophenyltrimethylammonium antibody. After the antiprotein-carrier antibody was removed by the addition of 20 μg of bovine serum albumin N/ml, the specific antibody was precipitated with azofibrinogen at concentrations ranging from 10 to 12 μg N/ml. The immune complex was dissolved in an excess of *p*-nitro derivative of the hapten and the mixture was chromatographed on a DEAE-cellulose column under the conditions described above. Two modifications were found necessary. First, the fibrinogen was pretreated with iodoacetic acid under conditions in which only the lysyl residues were modified. The increase in net negative charge achieved by carboxymethylation permitted the fibrinogen to be retained on DEAE-cellulose even after extensive coupling to the positively charged hapten. Second, an additional passage through carboxymethyl cellulose was employed to separate the mixture of antibody and positively charged hapten obtained from the DEAE-cellulose chromatography. When the cation exchange resin was equilibrated with 0.02 *M* phosphate buffer, pH 7.2, the antibody appeared as a single peak at column volume.

Purity of Antibody Preparations.—Since the purity of the antibody preparations was critical to the evaluation of the results, it was assayed by three different methods. First, the binding capacity was measured by the equilibrium dialysis technique, using procedures that have been described previously.[7] The arsonic antibody was dialyzed against acetylated arsanilic acid in which the carboxyl carbon was radioactive. The ammonium antibody was dialyzed against *p*-nitrophenyltrimethylammonium chloride synthesized with C^{14} methyl sulfate by a modification of the method of Zaki and Fahim.[11, 12] The binding capacity was calculated from the amounts of radioactivity found on each side of the membrane at equilibrium and from the antibody concentration determined by micro-Kjeldahl nitrogen analysis. In the six preparations tested, more than 1.95 of the theoretical maximum of two hapten molecules were bound per molecule of antibody. These values indicated that the preparations were pure within the limits of detection of the method.

Second, the molecular weights of both antibodies were determined by the equilibrium method of Yphantis.[13, 14] Two preparations isolated from a single rabbit were diluted to contain 1.0, 0.5, and 0.1 mg/ml, and equilibrated with 0.1 *M* phosphate buffer, pH 7.0, and 0.15 *M* KCl. The samples and buffer blanks were centrifuged at 20,410 rpm for 24 hr at 21.5°. The data from the 1 mg/ml solutions are shown in Figure 1, where the logarithm of the change in antibody concentration as measured by fringe height is plotted as a function of r, the distance in the cell from the center of rotation. In this method the linearity of the plots is an index of the purity of the material, and the excellent linear relationship shown in Figure 1 is consistent with a highly pure preparation.

Finally, the possibility that the samples contained small amounts of impurities introduced during the purification procedures was tested by the use of radioactive reagents. The antigens employed, bovine γ globulin, bovine serum albumin, and the azofibrinogens, were trace-labeled with two to four I^{131} atoms per molecule by a modification of the method of McFarlane.[15, 16] Specific haptens containing C^{14} were synthesized as described above for the equilibrium dialysis measurements. These radioactive components were then used at the appropriate stage in the purification procedure and the amount of radioactivity in the final product was

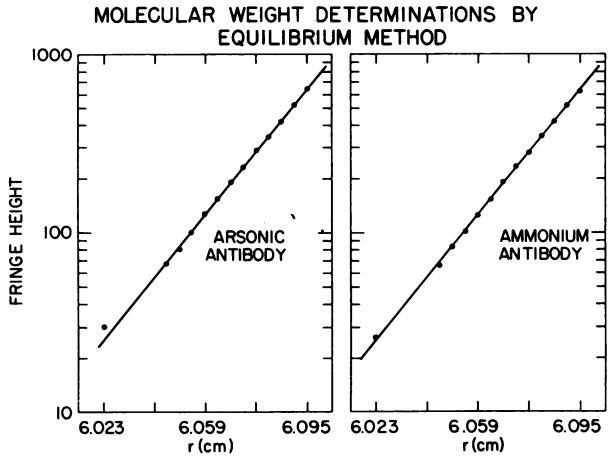

FIG. 1.—Determination of molecular weights by the method of Yphantis.[13] Since the fringe height at any point is directly proportional to the protein concentration, the measurement of fringe height at regular intervals in the cell gives the equilibrium distribution of material. The data shown in the left-hand plot were obtained with purified arsonic antibody at a concentration of 1 mg/ml. The data in the right-hand plot were obtained with purified ammonium antibody at the same concentration.

assayed. The results given in Table 1 show that the quantities of the respective protein antigens in the final products were negligible, less than 0.5% on a molar basis. The only significant contaminant was the five moles of *p*-nitrophenyltrimethylammonium chloride bound per mole of purified ammonium antibody. However, since at least 15 moles of each amino acid were recovered per mole of antibody, this amount of hapten contaminant represented only a 0.3% impurity in terms of the amino acid composition.

TABLE 1

EXTENT OF CONTAMINATION BY REAGENTS USED IN THE PURIFICATION PROCEDURE

Reagent	Ratio of Contaminant to Purified Antibody (moles/mole)	
	Arsonic antibody	Ammonium antibody
Protein carrier	3.0×10^{-4}	$<3.0 \times 10^{-7}$
Specific antigen	7.6×10^{-5}	6.3×10^{-5}
Specific hapten	$<1.0 \times 10^{-2}$	5.1×10^{-2}

Amino acid analyses: Amino acid analyses have been carried out on at least 15 different preparations of each antibody isolated from the pooled sera of immunized rabbits. The average recoveries obtained for each antibody after 20 hr of acid hydrolysis and the calculated standard errors of an individual determination are given in Table 2. The data have been expressed as moles of residue per mole of antibody and normalized to a leucine content of 89 and 91 residues for the arsonic and ammonium antibody, respectively. The values of 89 and 91 represent the average recoveries of leucine per mole of antibody as calculated from nitrogen analyses of the antibody hydrolysates and the assumptions of a nitrogen content of 16% and a molecular weight of 160,000.

To determine the total content of those amino acids known to be partially labile or resistant to 20 hr acid hydrolysis, samples of each antibody were hydrolyzed for periods of 20–96 hr. The threonyl and seryl residues in ammonium antibody were

TABLE 2

AVERAGE AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS* OF TWO PURIFIED ANTIBODIES ISOLATED FROM POOLED RABBIT SERA

Amino acid	Residues/160,000 g		Standard Error of a Single Determination	
	Arsonic antibody†	Ammonium antibody†	Arsonic antibody	Ammonium antibody
Lysine	71.4	68.7	1.66	2.13
Histidine	16.4	16.4	0.35	0.53
Arginine	45.3	41.9	1.19	1.36
Aspartic acid	105	111	0.72	0.73
Threonine	165	164	1.40	1.96
Serine	153	153	1.36	1.26
Glutamic acid	123	126	1.07	2.43
Proline	116	114	2.57	2.76
Glycine	110	111	1.41	1.08
Alanine	79.2	82.0	1.02	0.93
Half-cystine‡	43.7	44.1	0.50	0.72
Valine	127	129	2.93	2.61
Methionine	14.0	13.4	0.40	0.35
Isoleucine§	48.7	46.1	0.67	0.77
Leucine	89	91		
Tyrosine	55.9	56.5	1.54	0.83
Phenylalanine	44.2	45.4	0.56	0.41

* Hydrolysis time was 20 hr.
† Average values from the analyses of 13 different preparations.
‡ Determined as carboxymethylcysteine.
§ Sum of isoleucine and alloisoleucine.

found to be destroyed at the usual first-order rate, while those in arsonic antibody exhibited different labilities to acid hydrolysis.[16] However, extrapolation of the curves for each antibody to zero hydrolysis time gave identical values, 174 threonines and 172 serines. In addition to the losses in threonine and serine, the tyrosine recoveries were found to decrease slowly on longer hydrolysis, but even after extrapolation to zero hydrolysis time the average yield was 95% of that obtained from basic hydrolysates. No significant increases were observed in the valine and isoleucine recoveries upon prolonged hydrolysis, although Smith *et al.*[1] reported the incomplete release of these residues in 20-hr hydrolysates of rabbit antipneumococcal antibodies.

When the amino acid compositions of arsonic and ammonium antibody as given in Table 2 were corrected for the serine and threonine losses, and the tyrosine and tryptophan values from the basic hydrolysates[16] were substituted, the total residues recovered for each antibody were identical within experimental error (Table 3). Furthermore, the nitrogen content calculated from the total residues was found to be 16.4% for both antibodies. If these values were corrected for the 3–4% nitrogen present in the carbohydrate fraction, they were in excellent agreement with the assumed content of 16%, on which the determinations of the absolute leucine recoveries and the subsequent normalization procedure were based.

TABLE 3

PROPERTIES OF PURIFIED RABBIT ANTIBODIES

	Arsonic antibody	Ammonium antibody
Total residues recovered	1,458	1,465
% N in total residues*	16.4	16.4
Molecular weight at 1.0 mg/ml	163,500	163,500
Molecular weight at 0.5 mg/ml	163,500	164,500
Molecular weight at 0.1 mg/ml	159,600	160,300
Average molecular weight	162,200	162,800

* The calculation does not include the nitrogen in the carbohydrate fraction.

Further support for the amino acid compositions given in Table 2 was provided by the independent measurements of molecular weights. In Table 3 are listed the values calculated from the slopes of fringe height versus r, the experimentally determined density of the phosphate buffer solvent, and a partial specific volume for human γ globulin of 0.739.[17] The average of 162,200 for arsonic antibody and 162,800 for ammonium antibody not only demonstrated that the molecular weights of the two antibodies were identical within experimental error, but also justified the calculation of the amino acid compositions on the assumed value of 160,000.

Two features of the amino acid data were striking. The first was the over-all similarity in the compositions of the two antibodies. The second was the appearance of small, but significant differences. The arsonic antibody had a higher arginine and isoleucine content, while the ammonium antibody had a higher aspartic acid and leucine content. The significance of the difference in the arginine yields is not apparent from the standard errors shown in Table 2. However, the values given included not only the error of the analyses but also an additional pipetting error since the resolution of arginine required a separate application of the hydrolysate to the short column of the automatic analyzer. When a standard was used which was resolved on both the long and short columns or when the same pipette was employed for both applications, the error in a single arginine determination was reduced to less than 0.6 mole.

The observed differences, especially in the aspartic acid and arginine yields, were too large to be explained by the amounts of impurities found in the preparations. The possibility remained, however, that the results reflected genetic differences in the pooled sera from which the antibodies were obtained. This possibility was minimized by the data shown in Table 4, which represent the average amino acid recoveries from nine pairs of antibodies isolated from the sera of single rabbits. These analyses were performed under carefully controlled conditions; an assay of an amino acid standard was alternated with each assay of an antibody hydrolysate, and the errors in the recoveries on the short column were minimized

TABLE 4

AVERAGE AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS* OF TWO PURIFIED ANTIBODIES ISOLATED FROM THE SAME RABBIT

	Residues/160,000 g		Standard Error of a Single Determination	
Amino acid	Arsonic antibody†	Ammonium antibody†	Arsonic antibody	Ammonium antibody
Lysine	69.6	69.4	0.87	0.56
Histidine	16.4	16.6	0.23	0.16
Arginine	44.7	42.5	0.46	0.49
Aspartic acid	106	110	0.75	1.07
Threonine	162	162	3.05	2.53
Serine	151	151	1.90	1.70
Glutamic acid	125	127	2.15	2.00
Proline	109	110	2.74	2.35
Glycine	110	110	1.73	1.50
Alanine	81.1	81.4	0.86	1.65
Valine	128	128	1.80	1.70
Methionine	13.8	13.5	0.36	0.53
Isoleucine‡	48.4	46.4	0.93	0.96
Leucine	89	91		
Tyrosine	56.1	56.2	1.07	0.61
Phenylalanine	44.3	44.9	0.49	0.43

* Hydrolysis time was 20 hr.
† Average values from the analyses of nine different preparations.
‡ Sum of isoleucine and alloisoleucine.

by use of a single pipette for the application of the sample. It may be seen that the amino acid compositions of the two antibody preparations from individual rabbits were identical to those determined with pooled samples. Thus, the higher content of arginine and isoleucine in the arsonic antibody and the higher content of aspartic acid and leucine in the ammonium antibody were confirmed. Furthermore, using the standard statistical calculations, the differences in the arginine and aspartic acid values were shown to have a 99.9% probability of being outside experimental error.

Discussion.—The data presented above demonstrate for the first time significant differences in the amino acid composition of two purified rabbit antibodies. These results can be interpreted in two general ways. The first possibility is that the differences are related to antibody specificity and represent a change either in the contact amino acids at the active site or in residues which indirectly determine the steric arrangement of the active site. This interpretation would, of course, require that antibody synthesis be under genetic control and not result merely from a changed arrangement of the same peptide chains of γ globulin. The modification of the properties of a protein by a change in relatively few residues has already been demonstrated in the well-known studies of hemoglobin produced in sickle-cell anemia.[18, 19]

The second possibility is that the amino acid differences are not related to antibody specificity, but represent pools of different γ globulin molecules. The existence of γ globulin allotypes in rabbits has already been established by studies of their antigenic properties.[20–22] It is possible therefore that any one antibody activity is segregated or partially segregated in one type of globulin and that the amino acid differences merely reflect such a segregation. Since the same differences in composition were observed whether the antibodies were prepared from pooled sera or from the serum of a single animal, this interpretation requires that the two types of globulin are produced in all the animals used.

Support for the first hypothesis is provided by a correlation of the observed amino acid differences with the available evidence concerning the chemistry of the active sites. The higher aspartic acid content of the ammonium antibody is consistent with previous findings that its active center contains a negatively charged group. Grossberg and Pressman[23] showed that treatment of ammonium antibody with diazoacetamide destroyed the binding capacity and that the loss was prevented when the reaction was carried out in the presence of the homologous hapten. Analyses of the treated antibody indicated that the major change was a reduction in the number of free carboxyl groups. Similarly, the higher arginine content observed for arsonic antibody is consistent with the present chemical evidence for the structure of its active site. The pH dependence of the binding capacity indicated the participation of side chains with a pK of 10 or above. The presence of tyrosine, first suggested by Pressman and Sternberger,[24] was established by iodination and photooxidation studies in our laboratory.[25] The possible contribution of a lysyl residue was eliminated by Wofsy and Singer[26] who demonstrated that exhaustive amidination of the ϵ amino groups reduced the binding capacity by only 27%. These results were confirmed in our laboratory by the use of iodoacetic acid under conditions in which only lysyl residues were modified. It was found that 17% of the binding capacity was retained after carboxymethylation of all the ϵ amino

groups.[27] Since a single reactive tyrosine would not provide the degree of specificity observed for the arsonic antibody-antigen interaction,[7] the presence at the active site of the remaining positively charged determinant group, arginine, appears highly probable.

Further support for the first hypothesis is provided by genetic analyses of arsonic and ammonium antibodies isolated from the same rabbit. The results showed that the allotypic specificity of the two preparations was identical in every case although a variety of single and doubly heterozygous animals was represented. Furthermore, the allotypy of the purified antibodies corresponded with that of the whole serum except in two animals in which there was a deletion in both antibodies.[28]

The alternative hypothesis that the amino acid compositions reflect pools of different γ globulin molecules finds support in the considerable data indicating the microheterogeneity of both normal and immune γ globulin. In particular, the variation in the net charge among rabbit globulin molecules has been well established by use of zone electrophoresis and elution from carboxymethyl cellulose.[29, 30] These investigations have been extended to the univalent fragments liberated by papain, since they have been shown to differ in mobility in the same manner as the undigested globulin. Mandy and Nisonoff[31] have recently reported small differences in the charged amino acid content of the fragments which are in the direction expected from the mobilities. These data are consistent with the hypothesis that differences, at least in the charged amino acid recoveries, are not related to antibody specificity. However, Feinstein[30] found that the electrophoretic heterogeneity of γ globulin and its fragments could be accounted for by differences observed in the amide content rather than in the number of charged amino acid residues.

It is apparent from the above discussion that, although the finding of differences in the amino acid composition is suggestive, a definitive mechanism is not yet available. Amino acid analyses of several other antibodies, particularly one directed against an uncharged moiety, should help to distinguish between the alternative hypotheses. These experiments are in progress.

Summary.—Small but significant differences have been found in the amino acid composition of two antibodies purified from pooled sera or from the serum of the same rabbit. The antibody directed against the negatively charged *p*-azobenzenearsonic acid group had a higher arginine and isoleucine content, while the antibody directed against the positively charged *p*-azophenyltrimethylammonium ion had a higher aspartic acid and leucine content. The similarity in the recoveries of the remaining amino acids was striking, and the molecular weights of the two antibodies were shown to be identical within the limits of the method. The possible relationship of these differences in amino acid composition to antibody specificity and to the microheterogeneity of γ globulin is discussed.

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*PHYSICAL AND CHEMICAL PROPERTIES OF RNA FROM THE BACTERIAL VIRUS R17**

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In a previous communication[1] we have described R17, a newly isolated bacterial virus which contains RNA. The present report concerns properties of the purified viral RNA. It will be shown that R17 RNA can be obtained as a homogeneous, high molecular weight particle whose configuration in solution is a function of ionic strength and temperature.

Methods.—Purification of the virus: R17 virus was grown on *E. coli* K-12 (Hfr, methionine$^-$). It was purified by acid precipitation (of impurities), ammonium sulfate fractionation, and differential centrifugation as has already been described.[1] The purified virus was checked for infectivity by the agar layer procedure of Adams[2] and for homogeneity in the analytical ultracentrifuge with both schlieren and ultraviolet optics.

Isolation of R17 RNA: RNA was isolated from the virus by a phenol procedure