Differences in Amino Acid Composition of Rabbit γG-Antibodies of the Same Specificity*

James E. McGuigan,† Ernest S. Simms, and Herman N. Eisen

ABSTRACT: Rabbit anti-2,4-dinitrophenyl (anti-DNP) antibodies of different affinity for ε-DNP-L-lysine were compared with respect to their amino acid composition. Only those anti-DNP molecules of the γG-immunoglobulin class were analyzed, and all preparations were obtained from immunized rabbits that were homozygous at heavy- and light-chain loci (Aal,1-Ab4,4). In each of four animals the high-affinity antibodies made late in immunization differed from the low-affinity ones made earlier in the same rabbit by having about four more tryptophan and about four fewer alanine residues per molecule (assumed mol wt 160,000). In different rabbits the early–late-paired samples also differed significantly in one or more of a variety of other amino acids. Differences were also found among the anti-DNP preparations from different rabbits, even when isolated at the same time after immunization, when the average affinity for a reference ligand (ε-DNP-L-lysine) was essentially the same.

In accord with evidence that the specific reactivity of an antibody molecule is determined by its primary structure (Haber, 1964; Whitney and Tanford, 1965), significant differences in amino acid composition have been found among antibodies of different specificity (Koshland *et al.*, 1963, 1964, 1966; Bassett *et al.*, 1965). Antibodies of a given specificity can, however, differ considerably in their affinity for a particular ligand. The question arises, therefore, whether antibodies of the same specificity but different affinity are also demonstrably different in primary structure. In the present study, we have sought to answer this question by comparing anti-2,4-dinitrophenyl antibodies that differ in affinity for DNP ligands.

Since antibody molecules of a particular specificity may belong to different classes of immunoglobulins (Rockey *et al.*, 1964) the present analyses were limited to those of the γG-immunoglobulin class. Moreover, in order to minimize other structural differences that may be unrelated to specific binding activity, we compared in several rabbits the low-affinity and the high-affinity anti-DNP molecules isolated from the same animal; and all the rabbits used had the same immunoglobulin allotypes, and were homozygous at the known light- and heavy-chain loci (Oudin, 1966).

Our analyses show that among anti-DNP γG-immunoglobulins the low-affinity molecules made in a given rabbit differ in amino acid composition from those of high affinity formed by the same animal. Significant differences in amino acid composition were also found among the anti-DNP molecules isolated from different rabbits, even when they had similar average affinity for ε-DNP-L-lysine.

Materials and Methods

Antigens. Dinitrophenyl proteins were prepared and characterized as described (Eisen, 1964). Dinitrophenylated bovine γ-globulin, used for immunization, contained 56 moles of DNP/160,000 g of protein (DNP_{56}-BγG).[1] The protein used for analytical and preparative precipitin reactions with antisera was human serum albumin with 37 moles of DNP/70,000 g of protein (DNP_{37}-HSA).

Immunization. Ten New Zealand white rabbits, whose immunoglobulin allotype was 1,4 (genotype 1,1-4,4), were injected in their footpads with 5 mg of DNP_{56}-BγG in 0.8 ml of complete Freund's adjuvant.[2] The injections were repeated 6 months later. Blood was obtained by cardiac puncture 14 days after the first injection and 14 days after the second. Antibodies were isolated from sera of the first and second bleedings of the four rabbits with highest titers in their early bleedings. The purified antibodies from the early bleedings are referred to as "early antibodies," and those from the late bleedings as "late antibodies." Affinity for homologous hapten (ε-

* From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received December 6, 1967.* Part of this study was presented at the 50th Annual Meeting of The American Association of Immunologists (*Federation Proc. 25,* 677 (1966)) This work was supported, in part, by Research Grant AI-03231 and Training Grant 5 T1 AI-257, from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by Contract DA-49-193-MD-2330 with the Research and Development Command, Department of Defense, recommended by the Commission on Immunization of the Armed Forces Epidemiological Board.

† This work was performed during tenure of a special fellowship award from the National Institute of Allergy and Infectious Diseases (1-F3-AI-19,499).

[1] Abbreviations used not listed in *Biochemistry 5,* 1445 (1966), are: HSA, human serum albumin; BγG, bovine γ-globulin; Ea, chicken ovalbumin; TNP, 2,4,6-trinitrophenyl. Nomenclature and abbreviations for immunoglobulins are those recommended by the World Health Organization (*Bull. World Health Organ. 30,* 447 (1964)).

[2] We wish to thank Dr. Sheldon Dray for providing rabbits of established allotype (Aa 1,1-Ab 4,4).

TABLE I: Antibodies Subjected to Amino Acid Analyses.[a]

	"Early" Antibody				"Late" Antibody			
		Purified Antibody				Purified Antibody		
Rabbit	Level in Serum (mg/ml)	Recov[b] (%)	Av Intrinsic Assocn Constant[c] (K_0), M^{-1} ($\times 10^{-6}$)	Hetero-geneity Index (a)[d]	Level in Serum (mg/ml)	Recov[b] (%)	Av Intrinsic Assocn Constant[c] (K_0), M^{-1} ($\times 10^{-6}$)	Hetero-geneity Index (a)[d]
1	0.75	80	0.5	0.7	0.91	52	>100	0.3
2	0.42	88	1.1	0.8	1.73	49	>100	0.3
3	0.34	85	0.3	0.7	1.71	41	>100	0.3
4	0.46	81	0.8	0.7	0.92	46	>100	0.4

[a] Early antibody was isolated from serum collected 14 days after the first injection of the immunogen (DNP-BγG) and late antibody was isolated from serum collected 6 months later, 14 days after the second injection of immunogen. [b] Relative to amount of antibody precipitated from serum at equivalence by DNP-HSA. [c] For binding of ϵ-DNP-L-lysine. [d] Dispersion of association constants about the mean value, based on duplicate fluorescence titrations (Eisen and Siskind, 1964).

DNP-L-lysine) was relatively low in early and high in late antibodies (see Table I).

Purification of Antibody. Antibodies were isolated from each of the four early and four late sera. Serum samples were rid of complement by adding per milliliter: 40 μg of chicken ovalbumin (Ea) and 0.2 ml of a rabbit antiserum containing 280 μg of anti-Ea (Maurer and Talmage, 1953). After removal of the Ea–anti-Ea precipitate, the procedure was repeated; and after removal of the second Ea–anti-Ea precipitate, the anti-DNP antibodies were precipitated with DNP-HSA, extracted from precipitates with 0.1 M DNP-glycine (pH 8.5), and freed of DNP-HSA and of DNP-glycine by chromatography on Dowex 1 and DEAE-cellulose as described (Eisen *et al.*, 1968). Persistent traces of DNP-glycine, which remain associated particularly with high-affinity antibodies, were removed from aliquots used for measurement of affinity by exchanging 2,4-dinitrophenol for DNP-glycine on a Sephadex column, followed by removal of dinitrophenol on Dowex 1 (Eisen, 1964).

It was not possible to sacrifice enough of each sample to test for purity by the precipitin reaction or by equilibrium dialysis (to measure the number of binding sites per molecule). Nevertheless, it seems highly probable that there was no significant contamination by other proteins. First, each of the purified antibodies gave a single precipitin band of γG mobility when tested, at a concentration of about 4 mg/ml, by immunoelectrophoresis with a goat antiserum prepared against whole rabbit serum. Second, contamination by the antigen, DNP-HSA, was excluded by the conditions used for chromatography on DEAE-cellulose (Eisen *et al.*, 1968); any contaminating antigen would also be easily detected (and eliminated) by its quantitative precipitation in the final product, in which antibody would be in great excess. Third, replicate purifications of antibody from a single serum yielded samples whose amino acid compositions were identical (see below and Table II). Finally, extensive experience with the many other purified anti-DNP antibodies (of the γG class) obtained by the procedure used here has shown that they consistently have two ligand binding sites per molecule of 145,000–160,000 mol wt and are 90–95% specifically precipitable by DNP-HSA (see, for example, Eisen and Siskind (1964) and Little and Eisen (1966)).

Flourescence Quenching. Affinity of antibodies for DNP ligands was determined by fluorescence quenching (Velick *et al.*, 1960; Eisen and Siskind, 1964). Titrations were performed at 30° by adding 1–3 $\times 10^{-6}$ M ϵ-DNP-L-lysine to purified antibody at 40–80 μg/ml in 0.15 M NaCl–0.01 M KPO_4 (pH 7.4) (buffered saline).

Amino Acid Analyses. Each tube, containing 0.3–0.4 mg of antibody in 6 N HCl, was sealed after repeated freezing and thawing under vacuum and flushing with nitrogen. Hydrolysis was carried out in an oil bath at 110° for 24 hr. Amino acids were measured according to Spackman *et al.* (1958) with a Beckman Model 120B automatic analyzer equipped with scale expander and a 3.0-cm light path. Duplicate or quadruplicate hydrolysates were prepared from each sample of antibody, and single analyses for 15 amino acids were performed on each hydrolysate. Cystine and methionine values were uncertain and are not reported. Standard mixtures of amino acids, provided by the manufacturer, were analyzed repeatedly for calibration. Amino acid recoveries were normalized to 98 leucine residues/molecule of 160,000 mol wt, which represents the leucine content in a pool of early plus late anti-DNP antibodies derived from four other rabbits of 1,1-4,4 allotype; for this determination the amount of protein subjected to hydrolysis was estimated from nitrogen content, which was based on micro-Kjeldahl analyses, assuming 16% nitrogen. Although the molecular weight of rabbit γG-immunoglobulin is close to 145,000 (*e.g.*, Marler *et al.*,

1964), a molecular weight of 160,000 was assigned in this study in order to facilitate comparison with values in the literature for other rabbit γG-immunoglobulins (*e.g.*, Koshland *et al.*, 1963, 1964, 1966).

Tryptophan analyses were performed in triplicate or quadruplicate by Little's modification of the *N*-bromosuccinimide method (Patchornik *et al.*, 1960), which utilizes 6.7 M guanidine as denaturing agent in place of 6 M urea (Little and Eisen, 1968).

Statistical Analyses. Data for each of the 15 amino acids were subjected to analysis of variance (Snedecor, 1956). We are grateful to Dr. Reimut Wette and Mrs. Barbara Hixon of the Division of Biostatistics, Washington University School of Medicine, for discussions of the statistical methods and for carrying out these analyses. Computations were carried out on a digital computer at the Washington University Computing Facilities, supported in part by National Science Foundation Grant No. G222-96.

Results

Yield, Affinity, and Heterogeneity of the Samples. Yields of purified antibody ranged from 40 to 85% of the amount precipitable from serum by DNP-HSA. In accord with usual experience, affinity for the homologous hapten (ε-DNP-L-lysine) was much lower with the early than with the late antibodies, and the early samples were less heterogeneous with respect to affinity (Table I) (Eisen and Siskind, 1964; Fujio and Karush, 1966; Nussenzweig and Benacerraf, 1967; Parker *et al.*, 1967).

Replicate Purifications. Before comparing different samples it was necessary to consider whether the purification procedure itself was a source of variation. One serum sample, derived from a late bleeding, was divided into three parts, from each of which antibodies were isolated separately. The three preparations thus obtained were each hydrolyzed in duplicate, and analyzed for 15 amino acids. For 14 of the 15 there was no significantly greater variation among purifications than between the duplicate determinations of a given purification. Only serine showed a significant variation (at $P = 0.05$) among purifications, and this may be explained as a chance event expected in 1 of 20 analyses, or by the well-known lability of serine during acid hydrolysis (Moore and Stein, 1963). The values for some representative amino acids and for serine are given in Table II.

Early vs. Late Antibodies. The mean values for 15 amino acids in the early and late antibodies of four rabbits are shown in Table III, along with the number of replicate determinations and the standard deviations. Some pairs of early and late antibodies (each pair from a different animal) differed significantly (at $P = 0.01$) in one or more of the four animals in the following amino acids: histidine, arginine, aspartate, threonine, serine, glutamate, glycine, alanine, valine, isoleucine, tyrosine, phenylalanine, and tryptophan. However, the only early–late differences that were consistently significant in all four animals were in alanine and in tryptophan (Table IV).

TABLE II: Analyses of Some Representative Amino Acids from Replicate Purifications.[a]

Amino Acid	Purification 1	2	3
Glutamic acid[b]	141	138	139
	138	139	139
Proline[b]	117	118	118
	115	118	115
Valine[b]	140	138	140
	137	139	141
Serine	147	152	155
	155	154	156

[a] Replicate purifications were carried out on three aliquots of serum from one bleeding of one rabbit (12) obtained 14 days after a second injection of 5 mg of DNP-BγG in complete Freund's adjuvant, given 6 months after primary immunization with an identical injection. For each purification two hydrolysates were prepared and analyzed individually for 15 amino acids. The representative values shown are residues per molecule of protein (160,000 mol wt) normalized for leucine (see text). [b] Compare with variations in antibodies obtained from different rabbits (Table V).

Variations among Animals. Significant differences ($P \leq 0.001$) were also observed among the four early antibody samples with respect to lysine, histidine, threonine, serine, proline, valine, isoleucine, tyrosine, and phenylalanine; *i.e.*, the variations among means for these residues are significantly greater among early samples from different animals than between replicate determinations of individual samples. Values for some representative amino acids are shown in Table V. The late antibodies also differed significantly ($P \leq 0.001$) in different rabbits with respect to histidine, arginine, aspartate, threonine, serine, glycine, alanine, valine, isoleucine, and tyrosine.

Discussion

The antibody molecules of a particular specificity formed by a single animal are heterogeneous in a number of ways. They may, for example, fall into a variety of immunoglobulin classes and have different allotypes; and even those of the same class and allotype may differ greatly in affinity for a given ligand (combining site heterogeneity). In this study, we have been concerned particularly with the question of whether or not antibody molecules of the same specificity, immunoglobulin class, and allotype differ in primary structure when they differ in affinity for a given ligand.

Comparison of antibodies isolated from each of four rabbits shows that the high-affinity anti-DNP mole-

TABLE III: Amino Acid Composition of Early (E) and Late (L) γG Anti-DNP Antibodies from Four Rabbits (Allotype 1,1-4,4).[a]

		Rabbit No. 1			Rabbit No. 2			Rabbit No. 3			Rabbit No. 4		
		N	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD
Lysine	E	4	75.1	0.34	2	77.8	0.21	2	74.8	0.57	4	79.6	0.95
	L	4	74.6	3.67	2	74.5	0.14	2	71.0	.00	4	75.8	0.68
Histidine	E	4	18.7	0.18	2	16.8	0.07	2	14.2	0.21	4	21.0	0.36
	L	4	20.5	0.30	2	18.4	0.42	2	18.0	0.49	4	21.6	0.24
Arginine	E	4	45.3	1.18	2	45.7	0.57	2	45.2	0.42	3	47.1	0.35
	L	4	47.2	0.39	2	48.1	0.42	2	43.2	0.07	4	47.4	0.56
Aspartic acid	E	4	116	0.54	2	118	1.06	2	114	0.49	4	115	1.37
	L	4	114	1.30	2	115	0.00	2	111	0.64	4	116	0.43
Threonine	E	4	153	2.30	2	183	0.99	2	168	0.92	4	155	1.29
	L	4	162	2.06	2	178	0.42	2	165	0.35	4	157	0.97
Serine	E	4	153	1.59	2	161	0.35	2	155	0.85	4	139	1.12
	L	4	148	1.16	2	153	0.57	2	145	1.91	4	136	1.73
Glutamic acid	E	4	145	2.71	2	138	0.07	2	137	2.97	4	139	1.87
	L	4	139	2.45	2	136	1.56	2	130	1.27	4	138	0.72
Proline	E	4	110	1.82	2	115	1.91	2	109	0.49	4	113	0.78
	L	4	112	2.59	2	118	0.92	2	111	0.14	3	113	1.47
Glycine	E	4	118	0.57	2	119	0.50	2	118	0.57	3	106	0.67
	L	4	114	0.92	2	116	0.71	2	117	0.99	4	107	0.90
Alanine	E	4	89.1	0.66	2	84.1	1.70	2	83.9	0.57	4	89.0	3.28
	L	4	83.8	0.50	2	80.2	0.42	2	80.6	0.28	4	85.0	0.34
Valine	E	4	133	1.25	2	144	0.21	2	139	0.92	4	130	1.34
	L	4	136	1.38	2	145	0.28	2	138	0.14	4	133	0.75
Isoleucine	E	4	46.8	0.05	2	50.8	0.14	2	48.4	0.28	4	46.0	0.29
	L	4	46.3	0.36	2	48.4	0.07	2	48.6	0.07	4	46.4	0.17
Leucine	E	4	98		2	98		2	98		4	98	
	L	4	98		2	98		2	98		4	98	
Tyrosine	E	4	54.0	3.47	2	65.1	0.71	2	64.7	0.28	4	54.0	0.70
	L	4	54.8	0.84	2	61.0	0.92	2	58.3	0.57	4	51.9	1.37
Phenylalanine	E	4	44.2	0.22	2	51.8	0.35	2	47.2	0.07	4	45.8	1.10
	L	4	46.0	0.79	2	48.8	1.63	2	48.2	0.92	4	45.6	0.66
Tryptophan	E	3	18.7	1.76	3	19.4	0.53	3	19.7	0.80	3	20.2	0.85
	L	3	22.9	0.85	3	22.3	0.80	3	23.4	0.84	4	25.9	1.14

[a] *N* = number of hydrolysates analyzed. SD = standard deviation. The values were normalized to 98 leucine residues, except for tryptophan, which was measured with the intact protein. Mean values are mole residues per 160,000 g of protein.

cules made late after immunization differ from those of low affinity made earlier in the same animals by having, on the average, about four more tryptophan and four fewer alanine residues per molecule. A similar difference with respect to tryptophan has also been found between anti-DNP preparations (of the γG-immunoglobulin class) isolated from early and late pools of antisera obtained from large numbers of rabbits of diverse immunoglobulin allotypes (Little and Eisen, 1968; McGuigan and Eisen, 1968). As is shown elsewhere, early (low-affinity) and late (high-affinity) rabbit anti-DNP antibodies of the γG-immunoglobulin class have the same number of tryptophan residues per Fc fragment and per light chain, but the differences between the intact molecules were clearly evident in their respective Fab′ fragments (McGuigan and Eisen, 1968). Hence, the additional tryptophan residues of the late antibodies are probably located in the Fd′ piece. These observations are consistent with the view, based on a variety of lines of evidence, that amino acid sequence in the N-terminal halves of immunoglobulin chains (Hilschmann and Craig, 1965; Titani *et al.*, 1965), perhaps especially of the heavy chain (Fd piece) (Fleischman *et al.*, 1962; Utsumi and Karush, 1965; Haber and Richards, 1966), determine the reactivity of an antibody molecule's active sites.

In their careful studies of the distinctive amino acid differences among rabbit antibodies of different spec-

TABLE IV: Consistent Differences in Some Amino Acids between Early and Late Antibodies from Individual Rabbits.[a]

Amino Acid	Rabbit[b]	"Early" Antibody	"Late" Antibody
Alanine	1	89.5, 89.8, 88.7, 88.4	83.2, 84.4, 83.8, 83.6
	2	84.7, 84.1	79.9, 80.5
	3	83.5, 84.3	80.8, 80.4
	4	88.1, 93.9, 86.8, 87.4	85.1, 85.2, 84.5, 85.2
Tryptophan	1	16.7, 20.1, 19.2	23.0, 22.0, 23.7
	2	20.0, 19.2, 19.0	22.2, 21.6, 23.2
	3	19.6, 18.9, 20.5	23.8, 22.4, 24.0
	4	19.2, 20.5, 20.8	24.8, 27.4, 26.2, 25.2

[a] Each value for alanine represents a single determination of a separate 24-hr hydrolysate, normalized for leucine recovery (see text). Each value for tryptophan is a single determination by the *N*-bromosuccinimide method. Values are mole residues per 160,000 g of protein. [b] See Table I.

TABLE V: Values Showing Representative Variations among Amino Acid Residues of the Early Antibodies from Different Rabbits.[a]

	Rabbit No.			
Amino Acid	1	2	3	4
Glutamic acid	140	138	139	141
	146	138	135	139
	146			137
	146			138
Proline	108	114	109	114
	113	116	109	114
	110			112
	110			113
Valine	135	144	139	132
	132	144	138	131
	132			130
	133			129

[a] For these early antibody preparations, see Table I. Values are mole residues per 160,000 g of protein, normalized for leucine recovery (see Text). Each value represents a single determination on a separate 24-hr hydrolysate. Compare the variability among animals here with the constancy in replicate purified antibody preparations from a single serum of a fifth rabbit (12), shown in Table II.

ificities, Koshland *et al.* (1963, 1964) found that antibodies of the same specificity obtained from different rabbits were indistinguishable in amino acid composition; the antibodies examined were specific for *p*-azobenzenearsonate, or for *p*-azophenyl β-lactoside, or for *p*-azophenyltrimethylammonium. In contrast, we have found significant differences in composition among anti-DNP molecules from different rabbits, even when the antibodies were obtained at the same time after immunization and had essentially the same average intrinsic association constant for a reference ligand. If we make the reasonable assumption that the purified antibodies analyzed here and those examined by Koshland *et al.* were equally free of contaminating proteins, then it is necessary to consider why populations of anti-DNP molecules are more heterogeneous than those specific for one of several azophenyl derivatives.

We suspect that the difference stems largely from differences in the immunization procedures used in the two studies. Because the ensuing antibody production is especially rapid in onset, prolonged, and copious, we have used a single injection of DNP protein, or two widely spaced ones, in water-in-oil emulsion (plus mycobacteria; complete Freund's adjuvant). However, Koshland *et al.* (1963) used repeated injections of relatively large quantities of alum-precipitated azoprotein. The Freund's adjuvant method, in evoking a relatively greater response, probably stimulates the production of a greater diversity of immunoglobulins. It is also possible, however, as appears from the recent work of Haber and coworkers, that the antibodies elicited by certain immunogens are much less diverse than those elicited by others, even when similar determinant groups are involved and a similar immunization program is followed (Haber *et al.*, 1967; Richards *et al.*, 1967). It should be possible to evaluate the relative importance of these alternatives by comparison of the heterogeneity of antibodies formed in response to immunization with a protein in Freund's adjuvant and the same antigen in alum-precipitated form.

The recent findings of Nussenzweig and Benacerraf (1967) provide a basis for understanding some of the differences described here. In guinea pigs immunized with DNP proteins these investigators showed that a substantial minority (about 10–20%) of early low-affinity anti-DNP molecules have λ-type light chains, whereas in late high-affinity preparations almost all molecules have only κ-type light chains. That the light chains of rabbit immunoglobulins are also of κ and λ types has been recently shown (Hood *et al.*, 1967; Doolittle and Astrin, 1967), though this was not known when the present study was undertaken. Since κ and λ chains differ considerably in amino acid composition in their invariant regions (Putnam and Easely, 1965; Titani *et al.*, 1965), shifting proportions of these chains could account for at least some of the observed changes in amino acid composition with time.

Similar considerations could also account for differences in the anti-DNP antibodies isolated from different animals. The rate of transition from synthesis of low-affinity to high-affinity molecules varies somewhat in different rabbits (Eisen and Siskind, 1964; Steiner and Eisen, 1967), and it is possible that there are also individual differences in the rate at which there is a shift toward synthesis of molecules with a particular type of light chain. Even when two animals produce antibodies of the same average affinity, each of the populations is heterogeneous, and they need not have the same distribution of molecules with respect to affinity and light-chain type.

However, variations in light chains cannot account for all the time-dependent changes. For example, the additional tryptophan residues that distinguish late high-affinity anti-DNP molecules are located in the Fd part of the heavy chains (McGuigan and Eisen, 1968). As with light chains, it is also possible that variants of heavy (γ) chains, as in the γG-immunoglobulin subclasses of man, mouse, and other species (Grey *et al.*, 1965; Terry and Fahey, 1964; Warner and Herzenberg, 1966), are also present in the rabbit, though as yet unrecognized, and that varying proportions of these contribute to the observed differences in amino acid composition among anti-DNP γG preparations obtained at different times from the same rabbit, and at the same time from different rabbits. Whatever the precise reason for individual differences it is nevertheless clear that, unlike the findings with antibodies to several azophenyl derivatives (Koshland *et al.*, 1963, 1964, 1966), a given amino acid composition cannot be regarded as representative of rabbit anti-DNP antibodies of a given immunoglobulin class and allotype.

The finding that there are approximately four more tryptophans and four fewer alanines in the late antibodies raises the possibility that they occupy the same residue positions. It is doubtful that this possibility can be evaluated with antibodies of the kind examined in this study because, like most others obtained by current procedures, they are probably too heterogeneous for determination of extensive amino acid sequences in their variable regions.

References

Bassett, E. W., Tanenbaum, S. W., Pryzwansky, K., Beiser, S. M., and Kabat, E. A. (1965), *J. Exptl. Med. 122*, 251.

Doolittle, R. F., and Astrin, K. H. (1967), *Science 156*, 1755.

Eisen, H. N. (1964), *Methods Med. Res. 10*, 94, 120.

Eisen, H. N., Gray, W., Little, J. R., and Simms, E. S. (1968), *in* Methods in Immunochemistry and Immunology, New York, N. Y., Academic (in press).

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

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

Fujio, H., and Karush, F. (1966), *Biochemistry 5*, 1956.

Grey, H. M., Mannik, M., and Kunkel, H. G. (1965), *J. Exptl. Med. 121*, 561.

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

Haber, E., and Richards, F. F. (1966), *Proc. Roy. Soc. (London) B166*, 176.

Haber, E., Richards, F. F., Spragg, J., Austen, K. F., Vallotton, M., and Page, L. B. (1967), *Cold Spring Harbor Symp. Quant. Biol.* (in press).

Hilschmann, N., and Craig, L. C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1403.

Hood, L., Gray, W. R., Sanders, B. G., and Dreyer, W. J. (1967), *Cold Spring Harbor Symp. Quant. Biol.* (in press).

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

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

Koshland, M. E., Englberger, F. M., and Shapanka, R. (1966), *Biochemistry 5*, 641.

Little, J. R., and Eisen, H. N. (1966), *Biochemistry 5*, 3385.

Little, J. R., and Eisen, H. N. (1968), *Biochemistry 7*, 711.

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

Maurer, P. H., and Talmage, D. W. (1953), *J. Immunol. 70*, 135.

McGuigan, J. E., and Eisen, H. N. (1968), *Biochemistry 7*, 1919 (this issue; preceding paper).

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.

Nussenzweig, V., and Benacerraf, B. (1967), *J. Exptl. Med. 126*, 727.

Oudin, J. (1966), *Proc. Roy. Soc. (London) B166*, 207.

Parker, C. W., Godt, S. M., and Johnson, M. C. (1967), *Biochemistry 6*, 3417.

Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B. (1960), *J. Am. Chem. Soc. 82*, 5923.

Putnam, F. W., and Easley, C. W. (1965), *J. Biol. Chem. 240*, 1626.

Richards, F. F., Sloane, R. W., Jr., and Haber, E. (1967), *Biochemistry 6*, 476.

Rockey, J. H., Klinman, N. R., and Karush, F. (1964), *J. Exptl. Med. 120*, 589.

Snedecor, G. W. (1956), Statistical Methods, 5th ed, Ames, Iowa, Iowa State University, Chapters 10 and 12.

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

Steiner, L. A., and Eisen, H. N. (1967), *J. Exptl. Med. 126*, 1161.

Terry, W. D., and Fahey, J. L. (1964), *Science 146*, 400.

Titani, K., Whitley, E., Avogardo, L., and Putnam, F. W. (1965), *Science 149*, 1090.

Utsumi, S., and Karush, F. (1965), *Biochemistry 4*, 1766.

Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1470.

Warner, N. L., and Herzenberg, L. A. (1966), *J. Immunol. 97*, 525.

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