Different Distribution of Antibodies of Two Specificities Among γ-Globulins of an Individual Rabbit*

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Antibody directed against two different haptens was produced in an individual animal by simultaneous injection of two azoproteins, bovine γ-globulin-*p*-azobenzenearsonate (to give anti-R_p) and bovine γ-globulin-3-azopyridine (to give anti-P_3). The γ-globulin fraction from the antiserum of this animal was digested with papain and chromatographed by salt gradiant elution on carboxymethylcellulose. Assay of the eluted protein for anti-R_p and anti-P_3 activity indicated that most of the anti-P_3 activity appeared in subfractions of fraction I, whereas most of the anti-R_p activity appeared in subfractions of fraction II. This result lends strong support to the view that both univalent fragments of a given antibody molecule appear in the same chromatographic fraction. The data also lead to the interpretation that an individual can produce more than one type of antibody protein (7 *S* γ-globulin) directed against the same or different determinant groups.

When the γ-globulin fraction from the pooled antisera of several rabbits is digested with papain and chromatographed on carboxymethyl cellulose, three major fractions are obtained. Two of these (fractions I and II) contain univalent antibody fragments. The amounts of protein obtained in these two fractions have often been observed to be different, and each fraction often appears to be composed of more than one component (Stelos *et al.*, 1962). When specifically purified rabbit antibody from pooled antiserum is digested with papain and fractionated, even more widely disproportionate amounts in fractions I and II are frequently obtained (Grossberg *et al.*, 1962). These results have been interpreted as meaning that different kinds of antibody molecules are present in the γ-globulin preparations examined and that the antibody fragments which appear in one fraction are derived from one type of antibody molecule while those which appear in the other fraction are from another type of antibody molecule. This interpretation differs from that of Porter (1959), who suggested that the two univalent fragments of a single antibody molecule appear one in each of fractions I and II. Palmer *et al.* (1962) have also presented evidence that fragments appearing in fraction I are derived from molecules different from those whose fragments appear in fraction II.

Since all the above results were obtained with sera pooled from several rabbits, there remained the possibility that the multiplicity of antibodies observed was due to the pooling of sera. It was, therefore, important to know if the fragments obtained from the antibodies formed by a single animal would show similar heterogeneity. In the course of studying antibodies produced in single animals, we produced two different antihapten antibodies in an individual animal by simultaneous injection of two azoproteins. The results are reported here. The γ-globulin fraction from the serum of a single animal containing the two antibody activities was digested and chromatographed. Each antibody activity was found to be distributed unequally among components of fractions I and II of this chromatographed digest, and the pattern of distribution was not the same for the two specificities.

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MATERIALS AND METHODS

Preparation of Antigens.—Portions of bovine γ-globulin were coupled separately with diazotized *p*-aminobenzenearsonate and with diazotized 3-aminopyridine. The proportions of amines used were 0.25 mmole arsanilic acid and 0.27 mmole 3-aminopyridine per gram protein. The azoproteins were exhaustively dialyzed and made up to 20 mg/ml protein concentration in *p*H 8 borate-buffered saline. Equal volumes of the two solutions were mixed to provide antigen for injection.

Preparation of Antisera.—Rabbits were injected intravenously three times weekly on alternate days with 2 ml of the mixed antigen for a period of 3 weeks. One week after the last injection the rabbits were bled (20–25 ml serum) and the sera were titered for anti-R_p and anti-P_3 antibodies. The precipitating antigens used for this test were ovalbumin coupled with diazotized arsanilic acid (0.45 mmole amine/g protein) and ovalbumin coupled with diazotized 3-aminopyridine (0.54 mmole amine/g protein). Those rabbits (3 out of 10) having high titers of anti-R_p and also some titer of anti-P_3 were reinjected with 2 ml (20 mg) mixed antigen plus 10 mg bovine γ-gloublin-3-azopyridine. The rabbits were again bled after 1 week and then injected with antigen as previously, and a third blood sample was collected the following week. Only one rabbit showed a high titer of both antibodies, and the three portions of serum obtained as described were pooled.

Preparation of γ-Globulin Fragments.—The γ-globulin fraction of the serum from the single rabbit was prepared by the method of Kekwick (1940). γ-Globulin, 230 mg, was digested with 2.3 mg of papain at *p*H 7 for 1 hour at 37°. Ultracentrifugal analysis of a portion of the digest indicated that more than 95% of the protein had an s_{20} value of 3.5. Then 213 mg of the digested protein was dialyzed against 0.01 M *p*H 5.4 acetate buffer and chromatographed on a carboxymethylcellulose column (2.5 × 40 cm) equilibrated with the same buffer. After the initial portion of protein was eluted with 0.01 M buffer, the remaining protein was eluted by a concentration gradient generated as described previously (Stelos *et al.*, 1962), with 0.6 M *p*H

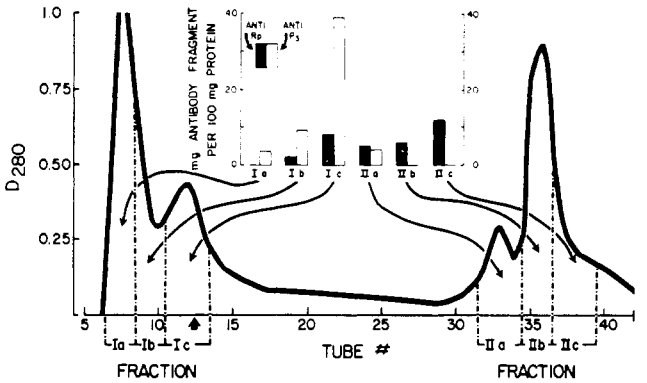

FIG. 1.—Distribution of anti-R_p and anti-P_3 activities in the fragments of γ-globulin from a single rabbit. The elution pattern shown does not include protein eluted later (fraction III), which did not contain antibody activity (see Methods). The arrow on the abscissa marks the point at which the gradient was begun. D_{280} values were obtained for each tube (10.3 ml); the exact shape of the solid curve could be deduced from a continuous record monitoring the per cent transmission at 280 mμ of the column effluent.

5.4 acetate as the limit buffer. The total amount of protein eluted was 190 mg (89% recovery); it appeared in three major heterogeneous fractions. The percentage of recovered protein in each fraction, designated in the manner originally described by Porter (1959), was fraction I, 36%: fraction II, 28%: fraction III, 36%.

Measurement of Binding of Hapten by Antibody.— Portions of the eluted protein appearing in fractions I and II were pooled according to the scheme diagrammed in Figure 1. Three subfractions of each of these fractions containing antibody activity were thus obtained. These solutions were concentrated by pervaporation and the content of anti-R_p and anti-P_3 binding sites and their average binding constants in each subfraction were determined by equilibrium dialysis. Similar determinations were made for the original intact γ-globulin. The haptens employed for binding measurements were I^{125}-labeled 3-iodopyridine (for anti-P_3) and I^{125}-labeled *p*-iodobenzenearsonate (for anti-R_p). These haptens were prepared in the same manner previously described for preparation of the I^{131}-labeled compounds (Grossberg *et al.*, 1962).

RESULTS

The elution pattern obtained on chromatography of the digested γ-globulin on CM-cellulose is shown in Figure 1. The protein eluted in the fraction I region consisted of at least two components separable under the conditions of chromatography employed, and the protein eluted in the fraction II region consisted of at least three separable components. The amounts of anti-R_p and anti-P_3 antibody in each of the isolated fractions are shown by the bars in Figure 1. These values were determined by extrapolating binding curves from measurements at three different hapten concentrations and taking the molecular weight to be 50,000 for univalent antibody fragments (Porter, 1959; Nisonoff *et al.*, 1960). The initial portion of protein eluted in fraction I contained anti-P_3 activity but no detectable anti-R_p activity, whereas the portions of fraction II eluted last contained anti-R_p activity, but no detectable anti-P_3 activity. Intermediate portions contained both activities. Almost all of the anti-P_3 activity was recovered in subfractions of fraction I, whereas the bulk of anti-R_p activity was recovered in subfractions of fraction II.

TABLE I
RECOVERY OF ANTI-R_p AND ANTI-P_3 ANTIBODIES IN FRACTIONS OF γ-GLOBULIN FRAGMENTS FROM A SINGLE RABBIT

	Fractions						
	Ia	Ib	Ic	IIa	IIb	IIc	Total
	Protein recovered (%)[a]						
	24	10	22[b]	9	18	17[c]	100
	Antibody activity recovered[a] (%)						
Anti-R_p	0	4	26[b]	7	19	39[c]	95
Anti-P_3	14	15	66[b]	9	0	0	101

[a] Protein recovered is on the basis of the total recovered, while antibody activity recovered is on the basis of the total activity present in the original amount of intact globulin. Antibody protein in intact γ-globulin for anti-R_p was 6.0 mg bivalent antibody (mw = 160,000) per 100 mg protein; anti-P_3, 7.8. [b] Includes protein eluted between fraction Ic and IIa (see text and Fig. 1). [c] Includes protein eluted after fraction IIc (see text and Fig. 1).

The per cent of antibody recovered in each fraction was calculated from the known antibody content of the original intact γ-gloublin. The protein eluted between fraction Ic and fraction IIa (Fig. 1) was taken to have the same amount of antibody per weight of protein as did fraction IIa. The remaining protein eluted immediately after fraction IIc was taken to have the same amount of antibody per weight of protein as did fraction IIc. The results of these calculations (Table 1) show that the recovery of antibody activity was essentially quantitative.

The average combining constants of the antibody sites recovered in each subfraction, compared to those of the intact γ-gloublin preparation, are given in Table II, together with the heterogeneity index for the distribution around the average values as calculated by the Sips equation (Sips, 1948). The combining constants for the antibodies of either activity were not the same for the different subfractions but were distributed around the values observed for the antibody in the intact γ-globulin preparation.

DISCUSSION

Antibodies formed in a single animal against a single antigen when digested by papain yield a variety of univalent active fragments which could be separated by chromatography on a CM-cellulose column. This type of separation has been observed previously with antibodies pooled from several rabbits (Stelos *et al.*, 1962). The present work shows that the presence of several fractions cannot be due to the pooling.

It was found previously that the amount of fragments in fraction I differs from that in fraction II, and this was the basis for the argument that different types of protein are involved as antibody and the active fragments from any one antibody molecule appear in the same chromatographic fraction. This argument

TABLE II
COMBINING CONSTANTS OF ANTIBODIES IN FRACTIONS OF γ-GLOBULIN FRAGMENTS FROM A SINGLE ANIMAL

	Intact γ-Globulin	Fractions					
		Ia	Ib	Ic	IIa	IIb	IIc
	$K_0 \times 10^{-5}$ (liter/mole)						
Anti-R_p	0.22	—	0.30	0.12	0.10	0.45	0.10
Anti-P_3	4.4	1.5	1.2	5.6	0.5		
	Heterogeneity Index(σ)						
Anti-R_p	0.6	—	1.0	1.0	0.6	0.8	0.6
Anti-P_3	0.6	1.0	1.0	1.0	1.0	—	—

depends on the demonstration that the disparity in the amounts of protein in fractions I and II is not due to an artifact of the digestion, i.e., the disproportionate amounts observed do not reflect random cleavage of peptide bonds by papain so that some of the molecules are split differently from others, yielding fragments which fall into fraction I or fraction II. The current results cannot be interpreted as an artifact of the digestion, for in that case the distribution of antibody activity in the various fractions would be the same for antibodies of both specificities.

The results do demonstrate that when antibodies formed against two different haptens are digested, the fragments of antibody of each specificity appear in the different fractions in unequal amounts. Furthermore, the distributions of the two specificities between the two major fractions are in opposite directions.

This cannot be an artifact of the digestion of a single kind of protein molecule. Our results show that more than one kind of protein molecule is involved for each specificity, because antibody activity of each specificity appears in more than one peak. The relative amounts of these proteins are different for the two specificities, thus giving rise to the observed differences in the proportion of anti-R_p to anti-P_3 in the various isolated fractions in the mixture studied.

A point of significance is that the antibody mixture described was from an individual rabbit; thus a single rabbit produces the various types of antibody protein.

The observed distribution of anti-R_p and anti-P_3 activity among the various proteins in this rabbit may well be different from the distribution in other rabbits, but the point of prime concern here is the demonstration that antibodies of different specificities are split differently to yield different fragments, as shown by their distribution between fraction I and fraction II.

Edelman *et al.* (1961) have also shown that antibodies of different specificities from an individual guinea pig appear to be largely of different kinds of protein, as shown by types of polypeptides produced on reduction of disulfide bonds.

It is known that the proteins associated with antibody function are very heterogeneous and appear in the γ-, β-, and macroglobulins. Even in the case of the 7 S γ-globulin proteins, it has been shown in mice (myeloma protein) (Fahey,1962) that each individual cell line produces its own type of γ-globulin protein. Each myeloma globulin was characterized by its own electrophoretic mobility and in many cases differed from others in its reaction with antibody directed against a particular gloublin.

A hypothesis (Pressman *et al.*, 1963) compatible with our results and with the above observations of others is that several different types of cells in a given animal produce antibody, each of a distinct protein type. Each cell can produce antibody against a multitude of substances, but with different degrees of efficiency. The type of protein which appears in the serum as antibody of a given specificity is determined by the types of protein produced by the cells which are most efficienct in producing antibody against the substance involved.

A single cell type could produce a multitude of antibodies from protein of a single primary structure, according to the theory proposed by Pauling (1940) in which a polypeptide chain of a particular amino acid sequence is folded somewhat differently to form antibodies of different specificities. Cells producing different sequences could each produce a multitude of antibodies by this folding mechanism. The individual binding sites would be formed similarly against a single antigenic determinant and combined in pairs to give the bivalent antibody. This is merely an amplification of Pauling's theory. All the 7 S γ-globulins have some common structural features such as the one that permits them to be split into three major portions by papain in the presence of reducing agent.

There remains the possibility that even two antibodies produced from a single cell and of protein with the same amino acid sequence could yield proteins of different properties not involving the specific site. The whole antibody molecule must necessarily differ for different antibodies as dictated by differences in the folding at the site. These differences in folding might well be too subtle to affect the chromatographic properties of fragments under the separation technique used.

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