Shape and Volume of Anti-Poly(D-alanyl) Antibodies in the Presence and Absence of Tetra-D-alanine as Followed by Small-Angle X-Ray Scattering†

Ingrid Pilz, Otto Kratky, Arieh Licht, and Michael Sela*

ABSTRACT: The conformation of two anti-poly(D-alanyl) antibodies was studied by small-angle X-ray scattering before and after interaction with hapten. With both samples a volume contraction could be observed upon interaction with the tetra-D-alanine hapten. The anti-poly(D-alanyl) antibodies obtained by immunization with poly(D-alanyl) diphtheria toxoid showed a decrease of the volume by 10% and a decrease of the radius of gyration by 7.7% when 90% of the binding sites were occupied by hapten. With anti-poly(D-alanyl) human serum albumin antibodies, a smaller decrease of the volume of 3.2% and of the radius of gyration of 1.4% was found, when 62% of the binding sites were occupied. Since the other data determined for the antibodies, such as molecular weight, radii of gyration of the cross-section, and form of the scattering curve, were unchanged upon interaction with hapten within the errors of measurement, it must be assumed that the volume contraction is due to a change in conformation upon interaction. The shape of all antibodies (free and occupied by hapten) is best described by a T-shaped model. The data found for the two antibody fractions, in the free and bound states, respectively, are the following: radius of gyration of the whole particle, a change from 6.08 to 6.00 nm (−1.4%) and from 6.50 to 6.00 nm (−7.7%); radii of gyration of the cross-section: R_{q_1}, from 2.31 to 2.43 and from 2.48 to 2.44 nm; R_{q_2}, from 1.40 to 1.46 and from 1.37 to 1.38 nm; volume, from 371 to 359 nm^3 (−3.2%) and from 399 to 358 nm^3 (−10%).

The interaction of the determinant group on the antigen with the combining site on the antibody represents a uniquely specific pattern of recognition on a molecular level. Antibodies are capable of inducing profound conformational changes in cross-reactive antigens, as exemplified by the conversion of a small synthetic periodic, not yet helical, polypeptide into an α-helical shape upon interaction with antibodies produced against a related ordered periodic polymer of a higher average molecular weight, which possessed under physiological conditions an α-helical structure (Schechter *et al.*, 1971). Similar observations were reported, concerning an arsanilated synthetic periodic polypeptide (Conway-Jacobs *et al.*, 1970) and a peptide derived from myoglobin (Crumpton and Small, 1967). To the same category belongs the reaction of metmyoglobin with anti-apomyoglobin resulting in the release of heme (Crumpton, 1966), the activation of an enzymatically inactive mutant of β-galactosidase (Rotman and Celada, 1968), stabilization of mutant catalase by complex formation with antibodies to normal catalase (Feinstein *et al.*, 1971), and the increase in RNase enzymatic activity upon adding anti-RNase antibodies to a mixture of S-protein and S-peptide, the two moieties obtained from RNase upon the cleavage of a single peptide bond by subtilisin (Cinader *et al.*, 1971).

It would be appealing to assume that, similarly to the induction of changes in antigen conformation by antibodies, transconformation might occur also within the antibody molecule as a result of its interaction with an antigen or a hapten. Moreover, in the case of an antigen, the steric change induced in the antibody might occur not only in the area vicinal to the combining site, but might also extend to other areas of the immunoglobulin molecule, especially when the possibility is taken into consideration that the cellular receptor for the antigen is of immunoglobulin nature, and that the triggering of the immune response is initiated by the reaction of an antigen with its receptor. The above hypothesis is in agreement with our knowledge of the structure of immunoglobulin, which is composed of several domains connected by flexible joints (Dorrington and Tanford, 1970; Nezlin *et al.*, 1970; Yguerabide *et al.*, 1970).

Many attempts to demonstrate changes in the gross conformation of the antibody molecule have been unsuccessful (Ishizaka and Campbell, 1959; Steiner and Lowey, 1966; Cathou *et al.*, 1969; Ashman *et al.*, 1971; Ashman and Metzger, 1971; Pecht *et al.*, 1972; Metzger, 1970). On the other hand, Grossberg *et al.* (1965) interpreted their studies on the effect of ligands on the rate of proteolysis of antibodies by chymotrypsin in terms of steric changes, even though other interpretations could be put forward (Metzger, 1970). Hapten can stabilize rather than change an antibody conformation (Cathou and Werner, 1970). Interesting studies were reported on a slight increase in the sedimentation coefficient of an antibody molecule upon its reaction with specific ligands (Warner and Schumaker, 1970a,b; Werner *et al.*, 1972). A possible interpretation was offered that the antibody molecule assumes a more compact configuration upon interaction with the hapten. Tumerman *et al.* (1972) reported recently an increase in the rotational relaxation time of an antibody molecule after reacting with its hapten, and drew the conclusion that conformational changes might lead to decreased flexibility. Marrack and Richards (1971) reported a kinetic study of the reaction of bovine serum albumin and its antibodies, and interpreted their results by assuming gross conformational

† From the Institute for Physical Chemistry, University of Graz, Graz, Austria (I. P.), Institut für Röntgenfeinstrukturforschung, Graz, Austria (O. K.), and The Weizmann Institute of Science, Rehovot, Israel (A. L. and M. S.). *Received June 8, 1973.* This study was supported in part by the "Österreichischen Fonds zur Förderung der wissenschaftlichen Forschung" and by Grant 06-010 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

changes upon the formation of large complexes. Liberti *et al.* (1972) reached a similar conclusion from a study of the change in tritium-exchange properties of a synthetic polypeptide antigen with its homologous antibodies.

The very recent report of Holowka *et al.* (1972) seems of particular interest. These authors have observed differences in the circular dichroism spectra of anti-S3-pneumococcal antibodies upon treating them with a specific hexasaccharide. Since the oligosaccharide alone shows no transitions in the wavelength range used, the spectral changes could be attributed to changes in the intrinsic optical activity of the antibodies. This finding could be interpreted as due either to conformational changes in the area of the combining site of the antibody or to a change in the environment of chromophores within the combining site due to the presence of the asymmetric hexasaccharide.

A powerful technique for the investigation of the structure of biopolymers in solution is the small-angle X-ray scattering; summarizing papers about this method are Kratky (1963), Kratky and Pilz (1972), and Pilz (1973). Already in 1950 Kratky *et al.* presented results on the overall shape of an immunoglobulin G. More recently, Pilz *et al.* (1970) have used this technique to investigate a homogeneous human immunoglobulin (Ig) myeloma protein of known primary structure. Their results suggested that the Fab and Fc regions of the molecule are relatively compact, but that the whole molecule has an extended structure in solution, fitting a T-shaped model in which the fragments are linked by a flexible hinge. This T-shaped model was confirmed by the first crystal X-ray study on an immunoglobulin G by Sarma *et al.* (1971). It seemed logical to use small-angle X-ray scattering technique also to the study of the changes, if any, occurring upon the reaction of an antibody molecule with its specific hapten.

In the present report we describe a small-angle X-ray scattering investigation of anti-poly(D-alanyl) antibodies in the absence and presence of the specific ligand, tetra-D-alanine. This particular system was selected as it has been investigated previously in detail (Schechter *et al.*, 1966, 1970; Schechter, 1971; Licht *et al.*, 1971), and it is known that the tetrapeptide is a complete antigenic determinant, *i.e.* that the antibody combining site is complementary to a peptide size of between three and four amino acid residues. The results described show clearly that the radius of gyration and the volume of the antibody molecule become smaller as a result of the interaction with the tetrapeptide.

Materials and Methods

Tetra-D-alanine, denoted $(D\text{-Ala})_4$ (Schechter and Berger, 1966), and poly(D-alanyl) human serum albumin (Schechter *et al.*, 1966) were gifts from Dr. I. Schechter. The syntheses of poly(D-alanyl) diphtheria toxoid (Licht *et al.*, 1971), of poly(D-alanyl) rabbit serum albumin (Schechter *et al.*, 1966), of the tetraalanine amide, $(D\text{-Ala})_4NH_2$, and of the radioactive tetrapeptide $(D\text{-Ala})_3$-[^{14}C]Gly (Schechter, 1971) were described previously. Poly(D-alanyl) human serum albumin had, on the average, 250 D-alanine residues attached per protein molecule, distributed among 32 chains, so that the average chain contained 7.8 D-alanine residues. Poly(D-alanyl) diphtheria toxoid had, on the average, 20 D-alanine residues attached per protein molecule, distributed among 2.8 chains, so that the average chain contained 7.1 D-alanine residues.

Normal rabbit immunoglobulin G was isolated from the serum by chromatography on diethylaminoethylcellulose (Levy and Sober, 1960).

Immunization. Rabbits were immunized in the foot pads by injecting 5 mg of antigen in complete Freund's adjuvant, followed by another two intramuscular injections, 10 and 20 days after the first immunization, of 10 mg of antigen. Sera from individual animals were collected at 1-week intervals and pooled for several months. The antigens used were poly(D-alanyl) human serum albumin and poly(D-alanyl) diphtheria toxoid.

Isolation of Antibodies. In the case of antisera against poly(D-alanyl) human serum albumin, the specific anti-poly(D-alanyl) antibodies were isolated by means of adsorption on a water-insoluble poly(D-alanyl) rabbit serum albumin–cellulose immunoadsorbent. This was prepared from poly(D-alanyl) rabbit serum albumin and bromoacetylcellulose (Robbins *et al.*, 1967) as described previously (Licht *et al.*, 1971). The adsorbed antibodies were eluted with 0.1 N acetic acid at 37° for 45 min.

For the isolation of anti-poly(D-alanyl) antibodies from antisera prepared against poly(D-alanyl) diphtheria toxoid, the IgG fraction was first obtained by repeating three times ammonium sulfate precipitation (respectively, at 40, 37, and 37% of the saturation) and solubilization in water. After dialysis against 0.15 M sodium chloride–0.01 M sodium phosphate (pH 7.4) the specific anti-poly(D-alanyl) antibodies were isolated with the immunoadsorbent described above. In both cases studies were performed on individual rabbits.

For the determination of antibody concentration, extinction values were obtained, based on Kjeldahl nitrogen analysis. The extinctions obtained were $E_{1\,cm}^{1\%} = 13.4$, for anti-poly(D-alanyl human serum albumin, and $E_{1\,cm}^{1\%} = 14.0$, for anti-poly(D-alanyl) diphtheria toxoid. The antibodies were equilibrated with 0.15 M sodium chloride–0.01 M sodium phosphate (pH 7.4) and concentrated by ultrafiltration. Determination of the sedimentation velocity in the analytical ultracentrifuge demonstrated that the antibodies isolated belonged to the IgG class.

Preparation of Antibody Samples for Small-Angle X-Ray Scattering. Both preparations were divided into two portions, one of which remained free of hapten, whereas the other one was saturated with the hapten $(D\text{-Ala})_4$ to a final concentration of the total hapten of 5×10^{-4} M in the case of antibodies raised with poly(D-alanyl) human serum albumin (62% of the antibody combining sites were occupied by the hapten), and with the hapten $(D\text{-Ala})_4NH_2$ to a final concentration of the total hapten of 1×10^{-3} M in the case of antibodies raised with poly(D-alanyl) diphtheria toxoid (90% of the antibody combining sites were occupied by the hapten). In the present paper the antibody raised with poly(D-alanyl) human serum albumin is referred as antibody I and the antibody raised with poly(D-alanyl) diphtheria toxoid is referred to as antibody II.

Small-Angle X-Ray Scattering Measurements. The small-angle measurements were made in a room of constant temperature and humidity with a highly stabilized X-ray generator using a copper tube. A camera (Kratky, 1958) with slit collimation was used. The experimental curves, therefore, include the collimation effect; they are slit-smeared. This is indicated by a bar (*e.g.*, $\bar{I}$, $\bar{R}$), whereby $\bar{I}$ means the slit-smeared intensity and $\bar{R}$ the value of the apparent radius of gyration calculated directly from the experimentally obtained, slit-smeared scattering curves. The collimation effects caused by the line shape of the primary beam were eliminated using a computer program previously described (Glatter, 1972).

A programmable step scanning device (Kratky and Kratky, 1964; Leopold, 1965) allowed automatic operation. A pro-

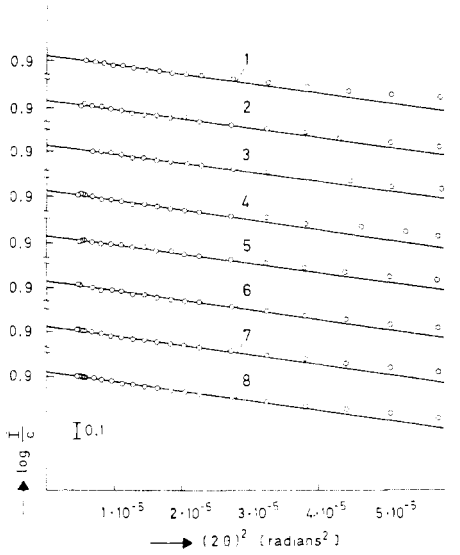

FIGURE 1: Innermost portions of the slit-smeared scattering curves of anti-poly(D-alanyl) human serum albumin (antibody I) in solution in a Guinier plot. The concentration (c) of the antibodies in solution and the apparent radii of gyration ($\bar{R}$) calculated from the slopes of the straight lines are summarized in Table I; the numbers in the figure correspond to the numbers in the table; I = scattered intensity; 2θ = scattering angle.

portional counter with pulse-height discriminator was used as a detector for Cu Kα lines. Elimination of the Kβ line was effected by using a computer program (Zipper, 1969).

The solutions of the antibodies were filled in Mark capillaries and irradiated at a temperature of 5°. The scattered intensities were measured at 90 different scattering angles in an angle range between 0.0013 and 0.14 radian. Each single solution was exposed to X-rays for 8–12 hr. During this time its scattering curve was recorded several times, whereby as a rule 10^5 pulses were registered for each measuring point of the curves of the antibody solutions and the blank curve of the corresponding buffer. The amount of the blank scattering was 20% at small angles for an antibody solution of a concentration of 30 mg/ml. The evaluation of the scattering data was done using the computer program of Zipper (1972). Since no significant alterations in the scattering behavior of either the antibody, or the antibody saturated with hapten, were detected during exposure to X-rays, conformational changes due to radiation damage could be excluded.

Concentration series (5–30 mg/ml) were measured for all preparations. Measurement of each sample was repeated at least once after 1–2 weeks, rendering identical scattering curves in every case.

The scattered intensity was normalized to concentration 1 by dividing all intensities by the corresponding concentration. In all plots the normalized intensity I/c (or $\bar{I}/c$ in case of smeared-out curves) will be used.

Partial Specific Volume. For the determination of the molecular weight of the antibodies their partial specific volume has to be known. It was determined for each sample by means of a digital densitometer (Kratky *et al.*, 1969) at the same temperature (5°) and in the same buffer as used for the small-angle measurements. The concentrations were determined by ultraviolet absorption using the extinctions given above.

Theory. From the small-angle X-ray scattering curves the following data were calculated: radius of gyration R, radius of gyration of the cross-section R_c, volume V of the antibody particle in solution by using the invariant (Porod, 1951), and molecular weight M (Kratky, 1963; Kratky *et al.*, 1950). The equations used for these calculations have already been given in this journal (Pilz *et al.*, 1970).

TABLE I: Apparent Radii of Gyration $\bar{R}$ Calculated from the Slopes of the Guinier Straight Lines of Figures 1 and 2 (Free and Bound Antibody I) and Figures 4 and 5 (Free and Bound Antibody II).[a]

Antibody I					
	Free		Saturated with Hapten (62%)		
No. in Fig. 1	c (mg/ml)	$\bar{R}$ (nm)	No. in Fig. 2	c (mg/ml)	$\bar{R}$ (nm)
1	5.00	5.15	1	5.01	5.09
2	5.00	5.13	2	5.01	5.04
3	10.06	5.15	3	10.1	5.10
4	10.06	5.18	4	10.1	5.02
5	19.92	5.08	5	19.6	5.09
6	19.92	5.14	6	19.6	5.06
7	30.00	5.14	7	30.0	5.00
8	30.00	5.12	8	30.0	5.00
Antibody II					
	Free		Saturated with Hapten (90%)		
No. in Fig. 4	c (mg/ml)	$\bar{R}$ (nm)	No. in Fig. 5	c (mg/ml)	$\bar{R}$ (nm)
1	5.07	5.41	1	5.53	5.02
2	5.50	5.40	2	10.5	5.02
3	9.87	5.41	3	21.2	5.01
4	10.1	5.41	4	29.9	5.06
5	19.4	5.54	5	42.0	5.09
6	20.1	5.40			
7	20.1	5.41			
8	30.6	5.55			
9	43.5	5.53			

[a] c is the concentration of the antibodies in solution.

Results

Radius of Gyration. The apparent radii of gyration $\bar{R}$ were calculated from the slope of the innermost portions of the scattering curves in the Guinier plot [log $\bar{I}/c$ *vs.* $(2\theta)^2$] as shown in Figures 1 and 2.

Antibody I. Figure 1 gives the scattering curves of two series of measurement of the anti-poly(D-alanyl) antibodies raised with poly(D-alanyl) human serum albumin and Figure 2 gives two series of curves of the same antibodies with which 62% of the combining sites were occupied by the hapten. The apparent radii of gyration $\bar{R}$ calculated from the slope of straight lines shown in the figures are summarized in Table I. In Figure 3 the obtained $\bar{R}$ values are plotted *vs.* the corresponding concentrations; up to a concentration of about 20 mg/ml no concentration dependence was found. The differences between the obtained averages ($\bar{R}$ = 5.15 nm for the free antibody and $\bar{R}$ = 5.07 nm for the antibody having hapten bound) are above the experimental error; the radius of gyration of the antibodies, the combining sites of which are saturated with hapten to the extent of 62%, is slightly lower than that of the free antibodies.

Antibody II. Figures 4 and 5 show the analogous curves for the anti-poly(D-alanyl) antibodies raised with poly(D-alanyl)

TABLE II: Data for Antibodies I and II.[a]

	M	V (nm³)	$\bar{R}$ (nm)	R (nm)	R_{q_1} (nm)	R_{q_2} (nm)	$\bar{v}$ (5°)
Antibody I							
Free	150,000	371	5.14	6.08	2.31	1.40	0.740
62% saturation	152,000	359	5.07	6.00	2.43	1.46	0.746
Difference		−3.2%	−1.4%	−1.4%			
Antibody II							
Free		399	5.45	6.50	2.48	1.37	
90% saturation	159,000	358	5.06	6.00	2.44	1.38	0.746
Difference		−10%	−7.2%	−7.7%			

[a] Antibodies I and II are free or have 62 and 90% of the combining sites occupied by hapten, respectively. M = molecular weight, V = volume of the particle, $\bar{R}$ = apparent radius of gyration, R = radius of gyration of the particle, R_{q_1} and R_{q_2} = radii of gyration of the cross-sections, $\bar{v}$ = partial specific volume.

diphtheria toxoid; in Figure 4 the curves of the free antibody are given and in Figure 5 those where 90% of the antibody combining sites were occupied by the hapten are given. The concentrations of the solutions used and the values of the apparent radii of gyration obtained are also given in Table I. Figure 6 shows the values of the apparent radii of gyration $\bar{R}$ plotted against the concentration of the antibody. The radius of gyration of the antibodies which have 90% of the combining sites saturated with hapten ($\bar{R}$ = 5.06 nm) is significantly lower than that of the free antibodies ($\bar{R}$ = 5.45 nm).

The correct values of the radii of gyration R obtained after elimination of the collimation influence are given for all samples in Table II besides the average values of the immediately obtained apparent radii of gyration $\bar{R}$. As is seen from the table, the radius of gyration of antibody I decreases by 1.4% and that of antibody II by 7.7% when 62 and 90%, respectively, of the combining sites are occupied by the hapten.

Radius of Gyration of the Cross-Section. The cross-section curves of the four different samples (free and bound antibodies I and II) are shown in Figure 7. The curves are very similar to each other, and show a course typical for all immunoglobulin G (IgG) (Pilz *et al.*, 1970; Pilz, 1970). From earlier investigations on a myeloma IgG and its fragments Fab and Fc we know that the flat outermost portion of the cross-section curves, which corresponds to a radius of gyration of the cross-section of R_{q_1} = 1.4 nm, can be associated with the cross-section of the Fab fragment. The steeper innermost part of the cross-section curves, corresponding to a radius of gyration of R_{q_2} = 2.3–2.4 nm, is due to the thicker central part of the T-shaped model. Table II summarizes the radii of gyration of the cross-section for the various antibodies calculated from the different slopes of the straight lines. The small differences in the values are within the experimental errors since the R_q values of the antibodies can be determined only with clearly less accuracy than the R values of the whole particle. Thus, the cross-section curves of the free and bound antibodies I and II can be regarded as identical, differences being within the errors of measurement (see Figure 7).

Volume. Using Porod's (1951) invariant the volumes of the different particles were determined. The value of the invariant Q, whereby $Q = \int_0^\infty I(2\theta)^2 d(2\theta)$, was calculated in the following way: in an angle range of 0–0.11 radian, the integration was performed by using the Simpson rule. The tail end of the scattering curve showed a course oscillating around $k/(2\theta)^4$, and in the angle range 0.11–∞ the integration was performed analytically after determining the value of the constant k. The absolute accuracy of the method is not very high, whereas the relative accuracy, *i.e.* the comparison of

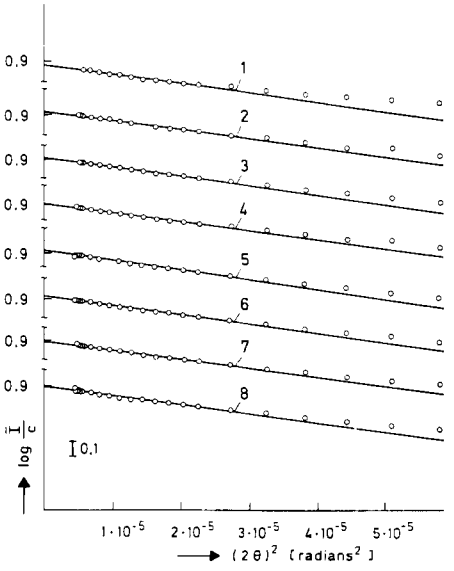

FIGURE 2: Innermost portions of the slit-smeared scattering curves of anti-poly(D-alanyl) human serum albumin (antibody I) after interaction with hapten; 62% of the combining sites of the antibody are occupied by hapten. The concentrations (c) of the solutions and the apparent radii of gyration ($\bar{R}$), calculated from the slopes of the straight lines, are summarized in Table I; the numbers in the figure correspond to the numbers in the table; I = scattered intensity; 2θ = scattering angle.

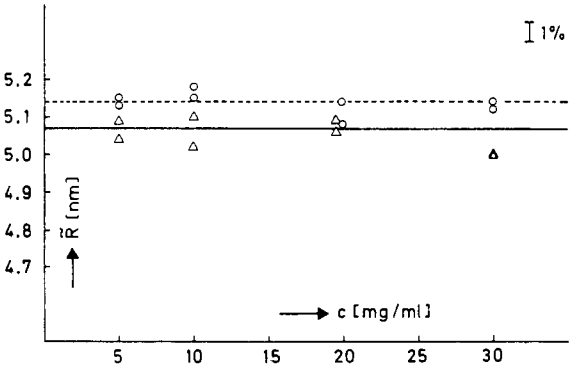

FIGURE 3: Apparent radii of gyration $\bar{R}$ plotted *vs.* the concentration (c) of anti-poly(D-alanyl) human serum albumin (antibody I) in solution; (○) values of the free antibodies; (△) values of the antibodies having 62% of the active sites occupied by hapten.

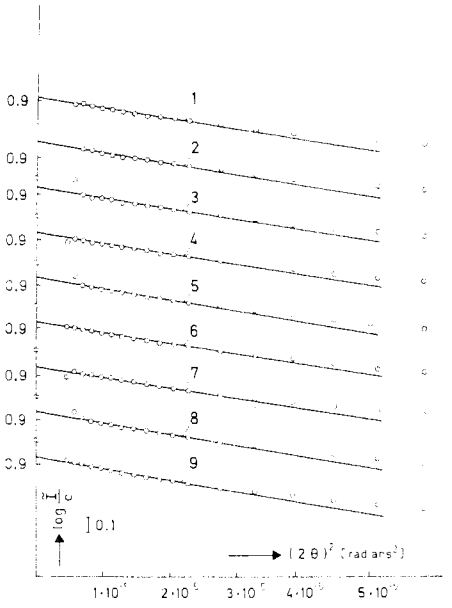

FIGURE 4: Innermost portions of the slit-smeared scattering curves of anti-poly(D-alanyl) diphtheria toxoid (antibody II) in solution in Guinier plot. The concentration (c) of the antibodies in solution and the apparent radii of gyration ($\bar{R}$), calculated from the slopes of the straight lines, are summarized in Table I; the numbers in the figure correspond to the numbers in the table; I = scattered intensity; 2θ = scattering angle.

the volumes of similar particles (as in the present case), is much better; the relative volumes can be determined with an error of 1–2%. It should be pointed out that the volumes determined in this way correspond to the hydrated particles including small voids.

The volumes for the various preparations are listed in Table II. It is striking that the occupancy of the combining sites of the antibodies with hapten is associated with a contraction, *i.e.* a decrease of their volume. This contraction amounts to 3% for antibody I (62% saturation) and 10% for antibody II (90% saturation).

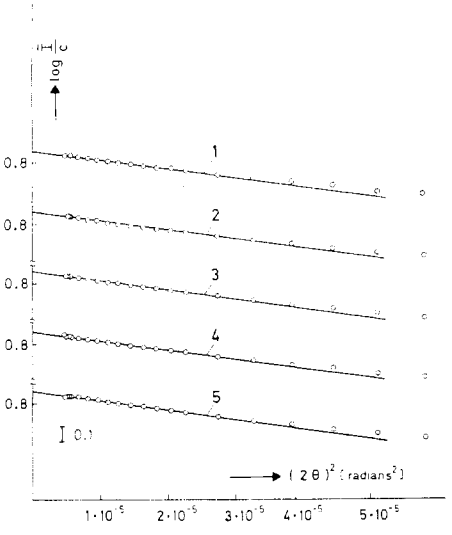

FIGURE 5: Innermost portions of the slit-smeared scattering curves of anti-poly(D-alanyl) diphtheria toxoid (antibody II) after interaction with hapten; 90% of the binding sites are occupied by hapten. The concentration (c) of the solutions and the apparent radii of gyration are summarized in Table I; the numbers in the figure correspond to the numbers in the table; I = scattered intensity; 2θ = scattering angle.

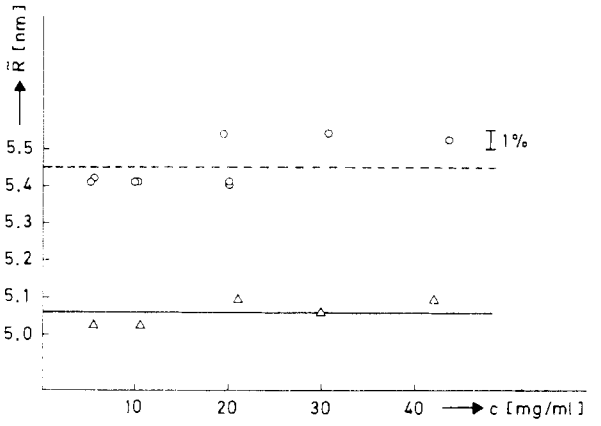

FIGURE 6: Apparent radii of gyration $\bar{R}$ plotted *vs.* the concentration (c) of anti-poly(D-alanyl) diphtheria toxoid (antibody II) in solution: (○) values of the free antibodies; (△) values of the antibodies having 90% of the active sites occupied by hapten.

Molecular Weight. The molecular weight as determined from small-angle X-ray measurements is very sensitive to errors in the concentration of the dissolved particle, since these errors also falsify to the same extent the values of the partial specific volume $\bar{v}$ determined by measuring the density of the solvent and the solution. We have determined the concentrations using optical densities as accurately as possible, and using these values we have measured and calculated the $\bar{v}$ values given in Table II. These values have been used to calculate the molecular weight given in the table too. Since an error of 1% in the determination of the concentration causes an error of 4% in the molecular weight calculated, the differences found in the molecular weight of the various samples are most likely the result of uncertainties in the concentration and therewith in the $\bar{v}$ values. Thus, the molecular weights ($M \simeq 150{,}000$) can be regarded unchanged to a first approximation, deviations being within the experimental error.

Particle Shape. The form of the scattering curves of all antibodies investigated is very similar and indicates that the particles have an anisotropic, elongated shape, which cannot be reproduced by any simple triaxial body. The typical shape of the cross-section curves (Figure 7) of all immunoglobulins gave a hint to the form of the particle. A comparison with theoretical cross-section curves of various models showed, as already described (Pilz *et al.*, 1970), that a T-shaped model (see Figure 8) fits best the typical cross-section curve.

In Figure 8 the experimentally obtained cross-section curve of free antibody II is compared with the theoretical curve of the model shown in the same figure. The model is built up from three cylindrical subunits which represent approximately the two Fab and the Fc fragments, respectively.

Volume, radius of gyration of the whole particle, and the two radii of gyration of the cross-section agree well with the experimental data. The shapes of experimental and theoretical curves are extremely similar. Thus, model b can be considered as a body equivalent in scattering. Its dimensions are: h_1 = 5.8 nm, a_1 = 2.4 nm, b_1 = 2.2 nm, h_2 = 11.9 nm, a_2 = 2.2 nm, b_2 = 1.8 nm. Upon the saturation of antibody II with hapten (90%) the volume decreases by 10% and the radius of gyration by 7.7% (Table II) without significant alterations in the shape of the cross-section curve (Figure 7). An attempt to account for those changes by altering model b of Figure 8 was made. Since the cross-section curve to a first approximation remains unchanged upon occupancy of the combining

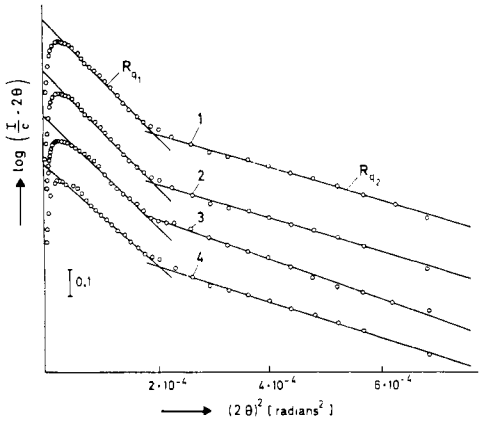

FIGURE 7: Cross-section curves in Guinier plot of the following samples: curve 1, anti-poly(D-alanyl) diphtheria toxoid (antibody II), 90% saturated with hapten; curve 2, free antibody II; curve 3, anti-poly(D-alanyl) human serum albumin (antibody I), 62% saturated with hapten; curve 4, free antibody I. The corresponding radii of gyration, R_{q1} and R_{q2}, calculated from the slopes of the steeper and flatter part of the curves, are summarized in Table II; I = scattered intensity in arbitrary units; 2θ = scattering angle.

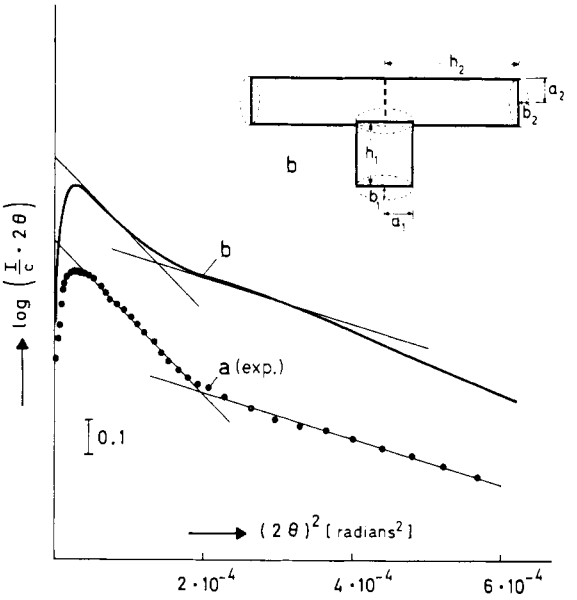

FIGURE 8: Cross-section curves in Guinier plot: curve a; experimental curve of free antibody II; curve b, theoretical curve of model b, consisting of three cylinders; the dimensions of the model are given in the text.

sites with hapten, the cross-sections of model b (Figure 8) have to stay the same. Thus, we have to assume that the decrease in volume and radius of gyration has to be accomplished mainly by shortening the long cylinders. By shortening the long cylinder, the necessary volume contraction (by 10%) and the decrease in the radius of gyration (by 7.7%) can be achieved without significantly changing the typical cross-section curve. The agreement of the experimental curve of antibody II with bound hapten (90%) was satisfactory.

Moreover, we examined what influence an additional bending of the cylinders around a hypothetical flexible joint might have on the cross-section curve. We found that the stretched T shape fits best the experimental curves of all antibodies, but a slight inclination of the Fab fragments to a very open Y shape cannot be excluded with certainty.

Discussion

The essential result obtained by investigating anti-poly(D-alanyl) antibodies in the presence and absence of the hapten tetra-D-alanine is that the occupancy of the specific binding sites of the antibodies by the haptens causes a clear contraction of the volume without significant changes in the type of shape.

Two anti-poly(D-alanyl) antibodies were investigated, anti-poly(D-alanyl) human serum albumin (designated as antibody I) and anti-poly(D-alanyl) diphtheria toxoid (antibody II). The volume of the free antibody I is 371 nm^3; its radius of gyration is 6.08 nm. By occupying 62% of the active sites by hapten, the volume (V) decreases by 3.2% and the radius of gyration (R) decreases by 1.4%. That this change of the R value is above the experimental error is demonstrated in Figure 3. With antibody II the effect of volume contraction is much more significant. Whereas the free antibody II has a volume (V) of 399 nm^3 and a radius of gyration (R) of 6.50 nm, the V value decreases by 10% and the R value by 7.7% after 90% of the binding sites are occupied by hapten.

There is a large difference in the extent of changes induced by a 62 *vs.* 90% occupancy of the combining sites by the hapten. This might be due to the fact that the anti-poly(D-alanyl) antibodies investigated were of a somewhat different nature, as they were obtained in different rabbits with different immunogens, and the antibodies investigated (I and II) differ by an order of magnitude in their affinity constants. Thus, antipoly(D-alanyl) antibodies, provoked with poly(D-alanyl) human serum albumin, bound (D-Ala)$_3$-[^{14}C]Gly with an association constant of $K_0 = 1.3 \times 10^6$ l./mol, whereas anti-poly(D-alanyl) antibodies provoked with poly(D-alanyl) diphtheria toxoid bound the same hapten with an association constant of $K_0 = 2 \times 10^7$ l./mol (Licht *et al.*, 1971).

Aside from the above considerations, there may be a significant difference due to single or double occupancy of the antibody sites. Thus, 62 or 90% saturation does not mean that there are 62 or 90% antibodies present in the solution which have occupied both binding sites by hapten. We have to expect that there is in solution a mixture of free antibodies, antibodies having only one active site occupied by hapten, and antibodies possessing both sites occupied by hapten. Assuming a statistical distribution, a saturation of 90% would mean that 81% of the particles would carry two haptens, 18% would carry only one hapten, and 1% of the antibodies would be free. A saturation of 62% would lead to the following partition: 39% of the antibodies occupied by two haptens, 47% by one, and 14% free antibodies.

As already mentioned, the changes could be observed mainly in a decrease of the volume and the radius of gyration of the whole particle; the other data, such as molecular weight ($M \simeq 150{,}000$), radii of gyration of the cross-section, and form of the scattering curve, are unchanged within the errors of measurement. It should be pointed out that the changes which are to be expected with all small-angle data, when the hapten is added to the antibody without leading to any changes in the conformation of the antibody, would be negligible because the molecular weight of the hapten used is only 350, that is 0.25% of the molecular weight of the antibody ($M \simeq 150{,}000$).

The shape of all free and bound antibodies investigated is best described by the T-shaped model (Figure 8, model b), which also fitted best the shape of the myeloma IgG1 investigated earlier (Pilz *et al.*, 1970). It is true that the stretched arrangement of the two Fab fragments gives the best agree-

ment, but a slight inclination of the Fab fragments cannot be excluded with certainty as the scattering curve of such a model does not differ very much from that of the stretched form. Studies on the shape of rabbit immunoglobulin G molecules in solution by using singlet–singlet energy transfer have also led to the conclusion that the molecule in solution has an open Y- or T-shaped conformation (Werner *et al.*, 1972).

Since the typical shape of the scattering curves and especially the cross-section curves was the same for free and bound antibodies it must be assumed that the change of the conformation of the antibodies upon interaction with hapten mainly consists in a change of the largest dimension, while the type of the shape (T shape or open Y) remains unchanged to a first approximation.

The dimensions of the models which are equivalent in scattering with the small-angle data are larger than the dimensions obtained by X-ray crystallographic studies (Sarma *et al.*, 1971), whereas the type of shape is the same. It must be assumed that IgG is a molecule which can have somewhat different dimensions in solution and in crystal.

A comparison of the data found in this study for anti-poly(D-alanyl) antibodies with those obtained earlier for myeloma IgG1 (Pilz *et al.*, 1970) and recently for anti-*p*-azophenyl β-lactoside antibodies (Pilz *et al.*, 1973) shows that there are also differences in the data obtained. The radius of gyration and the molecular weight of the myeloma IgG1 were higher, while its volume was smaller than those reported here for anti-poly(D-alanyl) antibodies. On the other hand, the radius of gyration of the anti-*p*-azophenyl β-lactoside antibodies agreed with that found for anti-poly(D-alanyl) antibodies. This difference might be related to the fact that the myeloma protein was of human origin, whereas the experimental antibodies were in both cases obtained in rabbits. The smaller radius of gyration, of the cross-section corresponding to the cross-section of the Fab fragment, is identical for all the immunoglobulins studied. The larger radius of gyration, of the cross-section corresponding to the middle part of the T-shaped molecule, varies somewhat. Summarizing all these data we may conclude that the overall typical T shape and the cross-section of the Fab fragments are identical for all IgGs, whereas there are some variations in the detailed structures.

The observation described in this paper, that the change in the gross conformation of the antibody molecule is due mainly to a volume contraction, seems to support the hypothesis (Edelman and Gall, 1969; Karlsson *et al.*, 1972) that the polypeptide chains of the immunoglobulins consist of globular regions between which there are less densely packed links. Small angle measurements on myeloma IgG1, and its fragments Fab and Fc, showed, too, that the volume and degree of swelling of the whole particle were clearly larger than the sum of the volumes of the fragments (2Fab + Fc) and their degree of hydration (Pilz *et al.*, 1970).

Ohta *et al.* (1970) reached from dilatometric measurements the conclusion that the antigen–antibody reaction is accompanied by an increase in volume, though a very small one. Our results contradict this conclusion. On the other hand, our finding that the antibody becomes more compact upon interaction with hapten would also agree with the interpretation of Warner and Schumaker (1970a,b) who assume, on the basis of sedimentation measurements, a more compact structure for the antibody after interaction with the hapten. Tumerman *et al.* (1972) assume from measurements of the rotational relaxation time also that there is a decrease of flexibility upon interaction with hapten. Further small-angle X-ray scattering studies with another antibody showed also a decrease in volume and radius of gyration after 50% of the active sites were occupied by hapten (Pilz *et al.*, 1973).

It seems that the changes in the antibody molecule resulting from its interaction with the hapten may be due either to a contraction involving the less densely packed regions of the molecule or to a decrease of flexibility in the "hinge" region. An investigation by small-angle X-ray scattering of the Fab and $(Fab')_2$ fragments of the antibody, in the presence and absence of hapten, may throw additional light on this problem.

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Preparation and Properties of the Repeating Sequence Polymer d(A-A-T)$_n$·d(A-T-T)$_n$†

Robert L. Ratliff,* D. Lloyd Williams, F. Newton Hayes, E. L. Martinez, Jr., and David A. Smith

ABSTRACT: The polymer d(A-A-T)$_n$·d(A-T-T)$_n$ has been prepared through a combination of organic chemical procedures and enzymatic synthesis with DNA polymerase from *Escherichia coli*. The physicochemical properties of the polymer have been compared with those of dA$_n$·dT$_n$ and d(A-T)$_n$·d(A-T)$_n$. Nearest neighbor analysis agrees with the assigned structure. Absorbance *vs.* temperature measurements as well as analytical buoyant density determinations in cesium chloride and cesium sulfate showed that d(A-A-T)$_n$·d(A-T-T)$_n$ has properties lying in between those of dA$_n$·dT$_n$ and d(A-T)$_n$·d(A-T)$_n$. Both strands of the d(A-A-T)$_n$·d(A-T-T)$_n$ polymer were found to be transcribed by the RNA polymerase from *E. coli* to give r(A-A-U)$_n$·r(A-U-U)$_n$.

The synthesis of polydeoxyribonucleotides has provided models for study of DNA and its biological functions. Single-stranded polymers with homo, multiblock, and random hetero sequences have been prepared by use of the terminal transferase from calf thymus (Bollum *et al.*, 1964; Ratliff *et al.*, 1967, 1968; Ratliff and Hayes, 1967; Kato *et al.*, 1967). Another procedure for obtaining single-stranded polydeoxyribonucleotides is separation of the complementary strands of double-stranded polymers (Wells and Blair, 1967). Double-stranded structures have been obtained by *de novo* synthesis, from primed reactions, and by the use of template. The *de novo* reactions using the DNA polymerase from *Escherichia coli* or *Micrococcus luteus* have yielded d(A-T)$_n$·d(A-T)$_n$ (Schachman *et al.*, 1960), dA$_n$·dT$_n$ (Burd and Wells, 1970), dC$_n$·dG$_n$ (Radding *et al.*, 1962), dC$_n$·dI$_n$ (Inman and Baldwin, 1964), and d(C-I)$_n$·d(C-I)$_n$ (Grant *et al.*, 1968). Primed amplification reactions have used complementary oligo- or polydeoxyribonucleotides that range from homo to repeating tetramer sequences (Wells *et al.*, 1965, 1967a,b, 1970; Morgan, 1970).

Seven of the possible repeating trinucleotide complementary double-stranded polydeoxyribonucleotides have been reported (Wells *et al.*, 1967a; Morgan, 1970). The five remaining are d(A-A-T)$_n$·d(A-T-T-)$_n$,[1] d(C-C-G)$_n$·d(C-G-G)$_n$, d(A-

† From the Cellular and Molecular Radiobiology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544. *Received August 22, 1973.* This work was performed under the auspices of the U. S. Atomic Energy Commission.

[1] The abbreviated notation for complementary double-stranded polydeoxyribotrinucleotides is in one of the two general forms allowed by the IUPAC–IUB Combined Commission on Biochemical Nomenclature (1970) and further incorporates an alphabetization rule that allows only one way to write the structure of each polymer. The left-hand member of the notation is stated as the alphabetically earliest three-letter set of the six possibilities in the polymer. The right-hand member is then fixed to be complementary in reverse order of letters.