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The Effects of Complete Modification of Amino Groups on the Antibody Activity of Antihapten Antibodies. Reversible Inactivation with Maleic Anhydride*

M. H. Freedman, A. L. Grossberg, and D. Pressman

ABSTRACT: The effect of reversible modification of free amino groups in the combining sites of antibodies directed against negatively charged haptens was studied. Two different pools of rabbit antisera directed against *p*-azobenzenearsonate (anti-R_p) were immunospecifically purified, and essentially 100% of their amino groups was selectively modified with maleic anhydride under conditions for which tyrosines are not modified. Both preparations lost a significant proportion, but not all, of their combining sites following complete maleylation of their amino groups. This loss of sites could be prevented by the presence of specific hapten during maleylation and could be reversed by hydrolytic removal of maleyl groups. A similar reversible loss of a proportion of the combining sites, following complete maleylation, was obtained with antibodies prepared against two other negatively charged haptens (*p*-azobenzoate and *p*-azobenzenephosphonate). The loss of antibody combining sites in anti-R_p antibodies and antibodies directed against *p*-azobenzenephosphonate was shown not to be due to any possible conformational changes caused by modification of amino groups outside of the antibody combining sites since loss of sites could be prevented completely by the presence of hapten during maleylation, and since the modification of all the amino groups in antibodies prepared against a neutral hapten (3-azopyridine) and against a positively charged hapten (*p*-azophenyltrimethylammonium) resulted in no change in either their number of antibody combining sites or their average binding constants. These results establish the presence of two kinds of chemically different combining sites in antibodies against negatively charged haptens, those containing an amino group and those that do not. In addition they establish the absence of amino groups in the combining sites of antibodies prepared against a positively charged and a neutral hapten.

Antibodies directed against several negatively charged haptens have a positive charge in their combining regions as demonstrated by the specific binding activities of various inorganic anions (Pressman *et al.*, 1961). The presence of free amino groups in the combining sites of some antibodies prepared against two negatively charged haptens (*p*-azobenzenearsonate and *p*-azobenzenephosphonate) was recently reported in studies utilizing the *N*-carboxyanhydride of DL-alanine (Freedman *et al.*, 1968). The failure to modify 100% of the amino groups in these antibody molecules with the alanine anhydride initiated the work reported in this communication.

Recently, Butler *et al.* (1967) reported on the reversible blocking of free amino groups in protein molecules using maleic anhydride. The authors suggested that the maleylation procedure was completely specific for amino groups. Maleic anhydride modifies essentially all of the amino groups present in protein molecules, and can be easily removed by acid hydrolysis.

This report describes the effect on antibody combining sites following chemical modification of all of the free amino groups in antibody molecules by maleic anhydride under mild conditions. The results demonstrate the direct involvement, in varying amounts, of amino groups in the combining sites of antibodies prepared against certain negatively charged haptens (*p*-azobenzenearsonate, *p*-azobenzenephosphonate, and *p*-azobenzoate), and the absence of amino groups in the combining sites of antibodies prepared against a positively charged (*p*-azophenyltrimethylammonium) and a neutral (3-azopyridine) hapten.

Materials and Methods

Buffers. The buffers used in this study included: Tris buffer (pH 8.0) (0.1 M Tris-HCl, 0.002 M EDTA), Tris-NaCl buffer (pH 8.0) (0.02 M Tris-HCl, 0.15 M NaCl, 0.002 M EDTA), and borate buffer (pH 9.0)

* From the Department of Biochemistry Research, Roswell Park Memorial Institute, Buffalo, New York 14203. *Received January 24, 1968.* Supported in part by Grant No. AI-02342 from the National Institute of Allergy and Infectious Diseases.

(10.3 g of H_3BO_3, 7.8 g of NaCl, and NaOH to adjust the pH to 9.0 per l.).

Preparation of Antigens. Azoproteins were prepared by coupling bovine γ-globulin (BGG)[1] with diazotized *p*-arsanilate to give BGG-R_p, with diazotized *p*-phosphanilate to give BGG-P_p, with diazotized *p*-aminobenzoate to give BGG-X_p, with diazotized *p*-aminophenyltrimethylammonium chloride to give BGG-A_p, and with diazotized 3-aminopyridine to give BGG-P_3 as described by Grossberg *et al.* (1962) and Kreiter and Pressman (1963).

Preparation of Antisera. The methods for preparing and testing rabbit antisera prepared against *p*-azobenzenearsonate (anti-R_p), *p*-azobenzenephosphonate (anti-P_p), *p*-azobenzoate (anti-X_p), *p*-azophenyltrimethylammonium (anti-A_p), and 3-azopyridine (anti-P_3) were described by Grossberg *et al.* (1962) and Kreiter and Pressman (1963).

Purification and Characterization of Antibody Preparations. Pooled rabbit antisera were the source of all antibody preparations used in this investigation. Anti-X_p, anti-A_p, and anti-P_3 antibodies were purified by the specific precipitation method described by Grossberg and Pressman (1968). Anti-R_p and anti-P_p antibodies were prepared by a single precipitation of the antisera with 50% saturated ammonium sulfate at 4°, followed by adsorption on specific solid immunoadsorbents (Onoue *et al.*, 1965). The adsorbed antibodies were eluted with 1 M propionic acid at 4° (Kitagawa *et al.*, 1967). Two different preparations (A and B) of specifically purified anti-R_p antibodies were used. All specifically purified antibody preparations were calculated to be greater than 90% antibody as determined by the binding of hapten (Grossberg and Pressman, 1960).

Preparation of Normal Rabbit Immunoglobulins. Normal rabbit immunoglobulins were prepared, from pooled normal rabbit sera, by three sodium sulfate precipitations at room temperature (Kekwick, 1940).

Maleylation of Rabbit Immunoglobulins and Purified Antibodies. In general, 0.1 mmole of solid maleic anhydride (Eastman, White Label) was added to 5 ml of protein solution (0.67×10^{-5} M) in borate buffer (pH 9.0) at 2° and maintained at pH 9.0 by the addition of 5 N NaOH. This is 43 moles of maleic anhydride/mole of lysine. The maleylation reaction was complete by the time the maleic anhydride had dissolved (5–10 min). The protected antibody sample, in most instances, contained 10^{-1} M hapten (final concentration) present before the addition of maleic anhydride. In the unprotected antibody sample, hapten was added to the same concentration, but after the maleylation reaction was completed. A control antibody sample was prepared simultaneously. It was adjusted to pH 9.0 and exposed to 10^{-1} M hapten, but maleic anhydride was not added. After maleylation of the protected and unprotected samples, all three preparations were exhaustively dialyzed for 4–6 days at 4°, first against 1000 volumes of Tris-NaCl buffer (pH 8.0) and finally against Tris buffer (pH 8.0). Before measuring the binding activities of the unprotected and protected maleylated antibody samples, all samples were always treated with hydroxylamine (0.8 M, pH 9.5). The extent of modification of the amino groups for the unprotected and protected samples was determined by measurement of their optical densities at 280 and 250 mμ.

Determination of the Extent of Amino Group Modification. The number of amino-bound maleyl groups was determined from optical density of the protein preparations (after appropriate dilutions with Tris buffer, pH 8.0) at 280 and 250 mμ before and after maleylation using the following as the molar extinction coefficients for rabbit immunoglobulins (219,000 at 280 mμ and 89,800 at 250 mμ for a molecular weight of 150,000), and using as the molar extinction coefficients for the maleylamino group, 310 at 280 mμ and 3360 at 250 mμ. The latter values were kindly supplied by Dr. B. S. Hartley[2] and were verified experimentally by determination of the optical density of known concentrations of ε-aminocaproic acid (Calbiochem) which had been treated with an excess of maleic anhydride under the conditions described above for the maleylation of proteins.

The number of maleyl groups per mole of protein is given by the expression

$$\frac{[(OD_{280\,m\mu})(89{,}800)] - [(OD_{250\,m\mu})(219{,}000)]}{[(OD_{250\,m\mu})(310)] - [(OD_{280\,m\mu})(3360)]}$$

where $OD_{280\,m\mu}$ and $OD_{250\,m\mu}$ are the values for the maleylated proteins.

The maleylation reaction when carried out as described above appears to maleylate all the lysyl groups in antibodies since the number of maleyl groups per molecule, calculated for a large number of preparations was always 68 ± 2, a number corresponding to the average number of lysyl residues per molecule of immunoglobulin or antibody as determined by amino acid analyses before maleylation. Amino acid analyses were performed on a Technicon automatic amino acid analyzer, using a 6.5-hr accelerated gradient,[3] with norleucine as internal standard. Moreover, there was no evidence for the presence of unreacted lysyl residues in the proteins after maleylation as shown by exhaustive dinitrophenylation using dinitrofluorobenzene as described by Lenard and Singer (1966). The absence of ε-*N*-(2,4-dinitrophenyl)lysine was checked in the hydrolysates using a Technicon amino acid analyzer.

Reaction of Maleic Anhydride with Protein Acyloxyl Groups. Maleic anhydride does not appear to react with hydroxyl-containing amino acid residues (tyrosine, serine, and threonine) under the above conditions, since subsequent treatment with 0.8 M hydroxylamine

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BGG, bovine γ-globulin; anti-R_p, anti-P_p, anti-X_p, anti-A_p, and anti-P_3, rabbit antisera directed against *p*-azobenzenearsonate, *p*-azobenzenephosphonate, *p*-azobenzoate, *p*-azophenyltrimethylammonium, and 3-azopyridine.

[2] Personal communication.

[3] Instruction manual AAA-1 for Technicon amino acid analyzer, p 38.

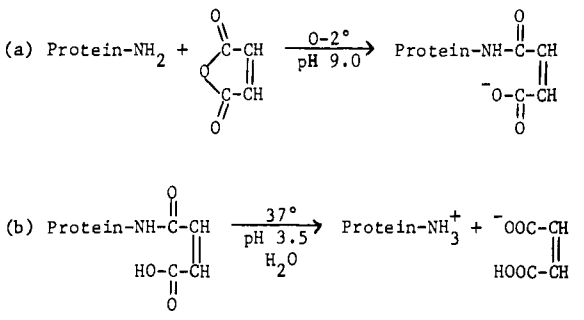

FIGURE 1: Reversible modification of amino groups. (a) The modification of protein amino groups with maleic anhydride at 0–2° in borate buffer (pH 9.0). (b) The hydrolysis of amino-bound maleyl groups at 37° in citrate–phosphate buffer (pH 3.5).

(pH 9.5) does not change the extinction values of the maleylated protein. However, more intensive treatment does maleylate hydroxyl groups. Hydroxylamine has been used to remove acyloxyl groups from proteins (Grossberg and Pressman, 1963; Gounaris and Perlmann, 1967). Therefore, to assure that only *N*-maleyl groups were present the maleylated antibody samples were always treated with alkaline hydroxylamine to remove *O*-maleyl groups.

The degree of maleylation of protein hydroxyl groups was determined by treating the maleylated proteins with hydroxylamine (0.8 M, pH 9.5) for 15 min with stirring at room temperature, then overnight at 4°. The hydroxamic acid formed was measured by bringing the pH of the solutions to 1.4 with concentrated HCl, and adding 0.37 M $FeCl_3$ in 0.1 M HCl in the amount suggested by Hestrin (1949). The protein precipitate which formed was removed by filtration, and the optical density of the supernatant was measured at 540 mμ, 15 min after addition of the $FeCl_3$ solution. Quantitation in all cases was referred to a standard curve of the amount of hydroxamic acid formed when known amounts of maleic anhydride were treated with alkaline hydroxylamine at pH 9.5.

Removal of Amino-Bound Maleyl Groups from Modified Anti-R_p Antibodies. The amino-bound maleyl groups were hydrolyzed by dialysis for 48–96 hr at 37° against several changes of large volumes of 0.1 M citric acid, 0.01 M sodium phosphate, and 0.002 M EDTA buffer (pH 3.5). The samples were then adjusted to pH 7.0 with 5 N NaOH, and dialyzed against several changes of Tris buffer (pH 8.0) at 4°. The number of amino-bound maleyl groups still present was determined by measurement of the optical densities at 280 and 250 mμ.

Determination of the Antibody Activity by Equilibrium Dialysis. The antibody activity of all unprotected, protected, and control antibody preparations was determined by equilibrium dialysis using ^{125}I-labeled haptens at several different free hapten concentrations, as previously described by Grossberg and Pressman (1960). Binding experiments were all run in Tris buffer (pH 8.0) at 4°. The antibody concentration was usually 0.67×10^{-5} M, and the hapten concentrations ranged from 0.5 to 8.0×10^{-5} M. This concentration range

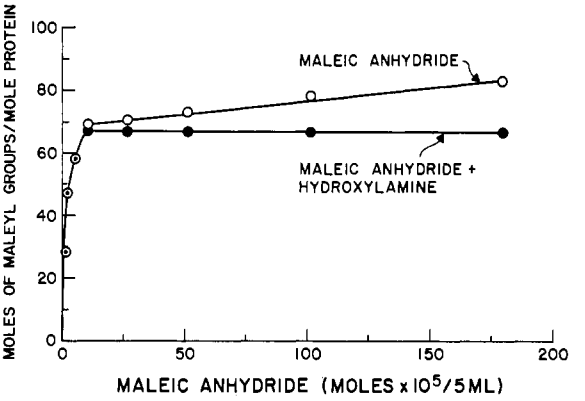

FIGURE 2: The extent of modification of normal rabbit immunoglobulins (0.67×10^{-5} M) with increasing amounts of maleic anhydride. (●) The number of *N*-maleyl groups per mole of protein after maleylation at pH 9.0 and subsequent treatment with alkaline hydroxylamine at pH 9.5 (○) The total number of maleyl groups per mole of protein after maleylation at pH 9.0 calculated as the sum of the *N*-maleyl groups (as calculated from the optical densities, see Methods section) and the *O*-maleyl groups (calculated as hydroxamic acid, see Methods section).

was selected so that the concentration range of free hapten would include that corresponding to half-saturation of the antibody. The use of the Sips equation and the associated methods of plotting the binding data were used to obtain values for the average binding constant (K_0), antibody site concentration (A_0), and heterogeneity index (a). The binding data were plotted according to the method described by Scatchard (1949), and first utilized by Karush (1956). The methods used to calculate the number of combining sites per mole of protein, the average binding constants, and the heterogeneity indices were most recently described by Freedman *et al.* (1968). The numbers of sites per mole of protein at infinite hapten concentration as plotted in the figures (open symbols) are the numbers derived from the above calculations. All binding values were corrected to the same protein concentration (0.67×10^{-5} M).

Preparation of ^{125}I-Labeled Haptens. ^{125}I-Labeled haptens (*p*-iodophenyltrimethylammonium, 3-iodopyridine, *p*-iodobenzoate, and *p*-iodobenzenearsonate) were prepared by isotope exchange utilizing carrier-free [^{125}I]iodide (Grossberg *et al.*, 1962). The exchange reaction to prepare [^{125}I]*p*-iodobenzenephosphonate was performed at 180° for 20 hr in a sealed tube, utilizing 20 μmoles of *p*-iodobenzenephosphonate and 3.0 mCi of ^{125}I in 0.5 ml of water at pH 4.5. The purified hapten had a specific activity of 250 mCi/mmole.

Sedimentation Analysis. Sedimentation velocity determinations were performed in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. The ultracentrifuge was operated at 59,780 rpm, and the rotor temperature was maintained at 20 ± 0.05°.

Determination of Protein. An $E^{1\%}_{1\,cm}$ of 14.6 at 280 mμ was used to determine the protein concentration of unmodified immunoglobulin and purified antibody preparations. Protein determinations of the protected,

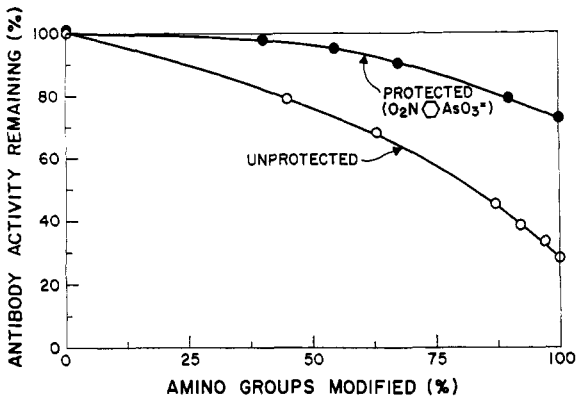

FIGURE 3: Curves showing the loss of binding activity of purified anti-R_p antibodies (preparation A) with increasing maleylation of amino groups. The loss of antibody activity is much less when modification of amino groups is carried out in the presence of 10^{-2} M *p*-nitrobenzenearsonate. Each point represents the mean of duplicate determinations with an average deviation from the mean of $\pm 0.8\%$. The activity was determined by equilibrium dialysis at a free hapten concentration of 7.7×10^{-6} M using ^{125}I-labeled *p*-iodobenzenearsonate. Antibody activity remaining expressed as "per cent binding activity," is defined as the concentration of hapten bound by a modified antibody preparation at a given free hapten concentration, divided by the concentration of hapten bound by an unmodified antibody preparation at the same free hapten concentration (the quotient multiplied by 100).

unprotected, and control antibody preparations following maleylation were performed using the Lowry modification of the Folin reaction (Lowry *et al.*, 1951).

Results

Maleylation of Normal Immunoglobulin and Specifically Purified Antibody Preparations. The reaction of

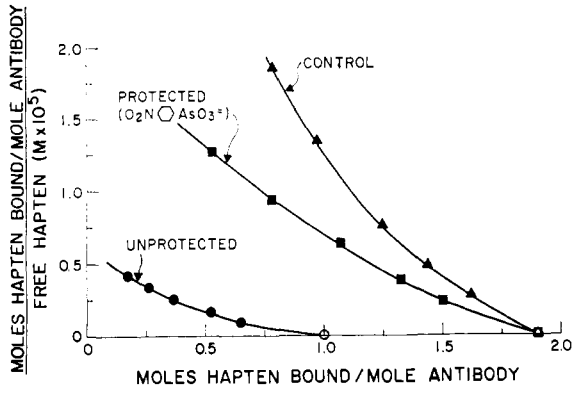

FIGURE 4: Binding curves demonstrating the loss of combining sites due to maleylation of all amino groups on the purified anti-R_p antibodies (preparation A) in the absence of hapten (●). No combining sites are lost, compared with the control antibody sample (▲), when the same extent of modification is carried out in the presence of 10^{-2} M *p*-nitrobenzenearsonate (■). Each point represents the mean of duplicate determinations with an average deviation from the mean of $\pm 1.0\%$. Open points (○, □, and △) on the abscissa are the values for the antibody sites present, estimated as described in the Methods section.

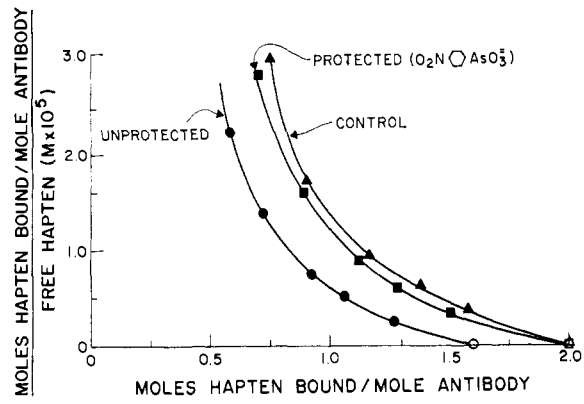

FIGURE 5: Maleylation of purified anti-R_p antibodies (preparation B). Maleylation conditions as in Figure 4. Each point represents the mean of duplicate determinations with an average deviation from the mean of $\pm 1.2\%$. Symbols as in Figure 4.

maleic anhydride with α or ϵ protein amino groups is illustrated in Figure 1. The number of maleyl groups attached to the protein molecules was dependent upon the amount of maleic anhydride used (Figure 2). The number of maleyl groups remaining after exposure of the modified protein to hydroxylamine was taken as the number of amino-bound maleyl groups. Any difference between the number of maleyl groups found before and after treatment with hydroxylamine was taken to represent the number of hydroxyl-bound maleyl groups. The number of amino-bound maleyl groups reached its maximum value (approximately 100% of the total number of lysyl residues present as determined by amino acid analysis) when 0.1 mmole of maleic anhydride was added to 5 ml of protein solution. When less than 0.1 mmole of maleic anhydride was added, no modification of hydroxylamino acids could be detected. As the amount of maleic anhydride was increased from 0.1 to 1.8 mmoles, the number of hydroxyl-bound maleyl groups increases almost linearly to 15 moles/mole of protein as determined by the hydroxamic acid method. Under the conditions described in the Methods section, essentially all the free amino groups were modified, and no hydroxyl-bound maleyl groups could be detected.

In the course of the maleylation of anti-R_p antibody with protection, it was observed that the hapten (*p*-nitrobenzenearsonate or benzenearsonate) when present at high concentrations, appears to react with the maleic anhydride.[4] The degree of maleylation of protein in the presence of these haptens was found to be less than in the absence of hapten apparently due to the competition of the hapten for the maleic anhydride.

The modification of amino groups in the protected and unprotected antibody samples was identical within

[4] Preliminary experiments were performed to determine the rate of hydrolysis of maleic anhydride at pH 7.0 in the absence and presence of 10^{-1} M haptens. The titrations suggested that the rate was accelerated in the presence of *p*-nitrobenzenearsonate and benzenearsonate haptens as compared with the rate of hydrolysis observed in the presence of all the other haptens tested including *p*-nitrobenzenephosphonate.

TABLE I: The Effect of Modification of All the Amino Groups in Anti-R_p Antibodies on the Combining Sites and Average Binding Constants.

Sample	Combining Sites[a] (per mole of protein)	K_0[b] × 10^{-5} (l./mole)	a[c]
Purified anti-R_p antibody (preparation A)			
Control	1.9	1.6	0.7
Protected[d] (10^{-2} M *p*-nitrobenzenearsonate)	1.9	0.8	0.8
Unprotected[d]	1.0	0.2	0.7
Purified anti-R_p antibody (preparation B)			
Control	2.0	1.0	0.6
Protected[d] (10^{-2} M *p*-nitrobenzenearsonate)	2.0	0.8	0.6
Unprotected[d]	1.6	0.9	0.6

[a] Values obtained by extrapolation of the binding curves as described in the Methods section. [b] Average binding constant. [c] Heterogeneity index. [d] Essentially all free amino groups were modified as determined by optical density measurements as well as by dinitrophenylation of the modified proteins as described in the Methods section.

the experimental limits of the assay procedure for all haptens used, except when anti-R_p antibodies were protected with 10^{-1} M *p*-nitrobenzenearsonate or 10^{-1} M benzenearsonate. When 0.1 mmole of maleic anhydride was added to 5 ml of these protected samples only 50–60% of the amino groups were modified. Since it is very important that the degree of modification in the unprotected and protected samples should be practically identical, different amounts of hapten and maleic anhydride were used to obtain essentially complete modification of anti-R_p antibody in the presence of hapten. The number of amino groups modified increased as the amount of maleic anhydride was increased from 0.1 to 1.3 mmoles for 5 ml of solution. At the higher amounts used, essentially all the amino groups were modified in the protected sample. This was also observed when the amount of maleic anhydride was maintained at 0.1 mmole, and the concentration of *p*-nitrobenzenearsonate was decreased from 10^{-1} to 10^{-2} M. Other haptens in a final concentration of 10^{-1} M had no inhibitory effect on the degree of modification of amino groups. These haptens included: phenyltrimethylammonium chloride,

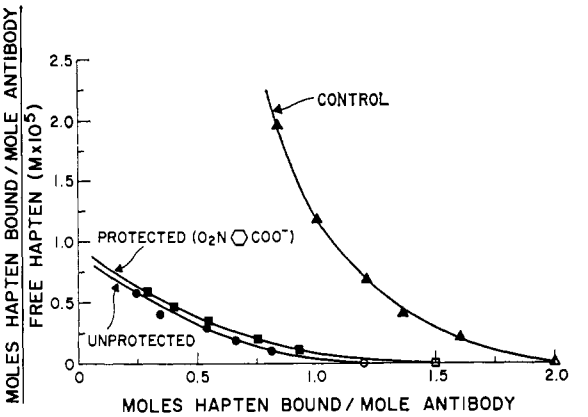

FIGURE 6: Binding curves showing the extent of loss of combining sites due to maleylation of all amino groups with purified anti-X_p antibodies in the presence and absence of 10^{-1} M hapten. Fewer sites are lost when the maleylation is carried out in the presence of 10^{-1} M *p*-nitrobenzoate. Each point represents the mean of duplicate determinations with an average deviation from the mean of ±1.1%. Symbols as in Figure 4.

pyridine, *p*-nitrobenzoate, benzenesulfonate, benzenephosphonate, and *p*-nitrobenzenephosphonate.

The Effect of Maleylation on Sedimentation Coefficients of Immunoglobulins. Untreated and maleylated immunoglobulin and antibody preparations were analyzed in the analytical ultracentrifuge. The unmodified preparations had an average $s_{20,w}$ of 6.6 S, while the maleylated samples had an average $s_{20,w}$ of 6.3 S. The apparent small reduction in the sedimentation coefficients of the samples after maleylation would seem to indicate that certain conformational changes occurred to cause a molecule with a slightly larger molecular weight than the original sample to sediment at a slightly slower rate. This is in accord with the observations of Habeeb *et al.* (1958) on succinylated proteins.

The Effect of Modification of Amino Groups on the Antibody Activity of Purified Anti-R_p Antibodies. In order to ascertain the effect of complete modification of amino groups, on the average binding constant, K_0, and on the number of antibody combining sites of antibodies made against negatively charged haptens, two different purified anti-R_p antibody preparations were modified with maleic anhydride in the presence (protected sample) and absence of *p*-nitrobenzenearsonate (unprotected sample). After extensive dialysis, the binding activity of the unprotected and protected samples was measured by equilibrium dialysis using ^{125}I-labeled *p*-iodobenzenearsonate. Results were compared with the binding activity of the control antibody preparation which was not treated with maleic anhydride but was otherwise carried through the same procedure.

Figure 3 demonstrates that increasing modification of the amino groups decreases the antibody activity of anti-R_p antibodies (preparation A), and the presence of hapten protects against this loss of activity. The increasing rate of loss of activity with degree of modification indicates that the important amino group being modified is one of lesser reactivity.

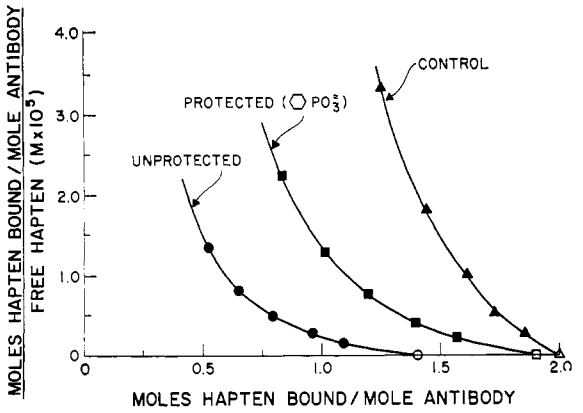

FIGURE 7: Binding curves demonstrating the loss of combining sites due to modification of all amino groups with purified anti-P_p antibodies in the absence of hapten. Essentially no combining sites are lost, compared with the control sample, when the same extent of modification is carried out in the presence of 10^{-1} M benzenephosphonate. Each point represents the mean of duplicate determinations with an average deviation from the mean of ±0.9%. Symbols as in Figure 4.

Binding curves for the unprotected, protected, and control anti-R_p antibody preparations A and B are illustrated in Figures 4 and 5, respectively, and the binding data are summarized in Table I. In all cases the number of amino groups modified was approximately 68 (±2) groups/molecule of protein, and no free lysyl residues could be detected by extensive dinitrophenylation. The nonlinearity of the binding plots given in Figures 4 and 5 reflects the heterogeneity of the antibody population with respect to its affinity for the *p*-iodobenzenearsonate hapten. Anti-R_p antibody (preparation A) (Table I and Figure 4) lost nine-tenths combining site per mole of antibody in the absence of hapten. The protected sample did not lose

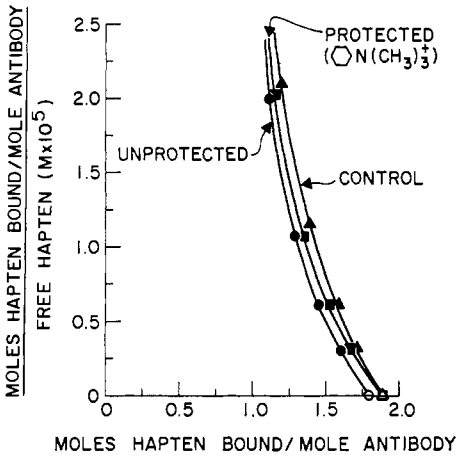

FIGURE 8: Binding curves showing no loss of combining sites and no change in their average binding constants following modification of all amino groups on purified anti-A_p antibodies. The protected sample contained 10^{-1} M phenyltrimethylammonium during maleylation. Each point represents the mean of duplicate determinations with an average deviation from the mean of ±0.9%. Symbols as in Figure 4.

TABLE II: The Effect of Modification of All the Amino Groups in Anti-X_p and Anti-P_p Antibodies on the Combining Sites and Average Binding Constants.

Sample	Combining Sites[a] (per mole of protein)	K_0[b] × 10^{-5} (l./mole)	a[c]
Purified anti-X_p antibody			
Control	2.0	1.3	0.6
Protected[d] (10^{-1} M *p*-nitrobenzoate)	1.5	0.3	0.6
Unprotected[d]	1.2	0.3	0.7
Purified anti-P_p antibody			
Control	2.0	6.4	0.7
Protected[d] (10^{-1} M benzenephosphonate)	1.9	1.8	0.7
Unprotected[d]	1.4	0.9	0.7

[a] Values obtained by extrapolation of the binding curves as described in the Methods section. [b] Average binding constant. [c] Heterogeneity index. [d] Essentially all free amino groups were modified as determined by optical density measurements as well as by dinitrophenylation of the modified proteins as described in the Methods section.

any antibody combining sites when compared with the control sample. The average binding constant in the unprotected sample was reduced approximately eightfold, while the average binding constant in the protected sample was reduced only twofold. The heterogeneity indices were practically identical for the unprotected, protected, and control samples. Anti-R_p antibody (preparation B) (Table I and Figure 5) did not show as great a loss of antibody combining sites as did preparation A, and the value of K_0 was not affected.

The Effect of Modification of Amino Groups on the Antibody Activity of Anti-X_p and Anti-P_p Antibodies. Anti-X_p and anti-P_p antibodies were similarly maleylated, and the binding curves are presented in Figures 6 and 7 and the binding data are summarized in Table II.

Maleylated anti-X_p antibodies lost eight-tenths combining site per mole of antibody when hapten was absent (unprotected sample), and five-tenths combining site per mole of antibody when hapten was present (protected sample). The average binding constant for both the unprotected and protected samples was reduced approximately fourfold when compared with the control sample.

The unprotected anti-P_p antibodies after maleylation lost six-tenths combining site per mole of antibody,

TABLE III: The Lack of Effect of Modification of All the Amino Groups in Anti-A_p and Anti-P_3 Antibodies on the Combining Sites and Average Binding Constants.

Sample	Combining Sites[a] (per mole of protein)	K_0[b] $\times 10^{-5}$ (l./mole)	a[c]
Purified anti-A_p antibody			
Control	1.9	4.3	0.7
Protected[d] (10^{-1} M phenyltrimethylammonium chloride)	1.9	3.7	0.7
Unprotected[d]	1.8	3.8	0.7
Purified anti-P_3 antibody			
Control	1.9	1.1	0.7
Protected[d] (10^{-1} M pyridine)	1.9	1.0	0.8
Unprotected[d]	1.9	1.0	0.7

[a] Values obtained by extrapolation of the binding curves as described in the Methods section. [b] Average binding constant. [c] Heterogeneity index. [d] Essentially all free amino groups were modified as determined by optical density measurements as well as by dinitrophenylation of the modified proteins as described in the Methods section.

while the protected sample lost essentially none The average binding constant of the unprotected sample was reduced approximately 7-fold after modification, and the average binding constant of the protected sample was reduced only 3.5-fold.

The Absence of Amino Groups in the Combining Sites of Anti-A_p and Anti-P_3 Antibodies. Specifically purified anti-A_p and anti-P_3 antibodies were also maleylated and showed no loss of activity. The binding curves are shown in Figures 8 and 9, and the binding data are given in Table III. There was essentially no change in the number of sites or K_0 for either antibodies even though 100% of the lysyl residues were modified.

The Effect of Removal of Amino-Bound Maleyl Groups on the Antibody Activity of Modified Anti-R_p Antibody. Recovery of activity was found to take place on hydrolysis of maleyl groups from maleylated anti-R_p antibody (preparation A). The binding curves for maleylated anti-R_p antibody before and after hydrolysis for 48 and 96 hr (Figure 10 and Table IV) show increasing recovery of activity with time of hydrolysis. By 96 hr, the recovery was almost complete since the antibody had the same number of antibody combining sites with almost the same K_0 value as the control unmodified antibody. Exposure of the unmodified antibody to acid pH and elevated temperature had essentially no effect on either the number of antibody combining sites or the K_0 value.

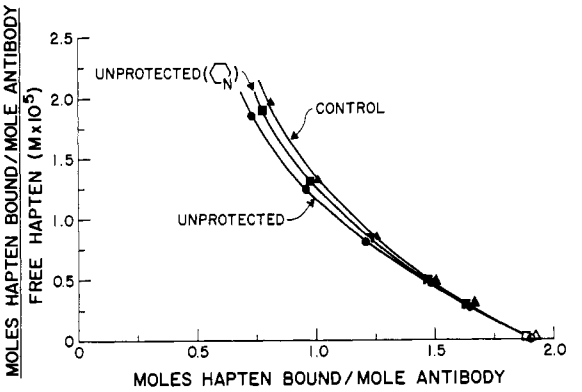

FIGURE 9: Binding curves demonstrating no loss of combining sites and no change in their average binding constants following modification of all amino groups on purified anti-P_3 antibodies. The protected sample contained 10^{-1} M pyridine during maleylation. Each point represents the mean of duplicate determinations with an average deviation from the mean of $\pm 1.2\%$. Symbols as in Figure 4.

The maleylated antibody precipitated when added to the citrate buffer (pH 3.5), but redissolved slowly as hydrolysis proceeded. At pH 6 or above, the modified proteins are very soluble since the negative maleyl groups increase electrostatic repulsion and decrease aggregation. At pH 3.5, the negative carboxylates are very largely converted into un-ionized carboxyls so that the electrostatic repulsion is greatly reduced causing aggregation and precipitation (Figure 1). As hydrolysis proceeds, the formation of excess positive charges probably again produces repulsion leading to solution. The control unmodified sample did not precipitate at any time during the hydrolysis procedure.

Discussion

The antibody preparations were usually modified at a protein concentration of 0.67×10^{-5} M, and the antibodies were treated with 0.1 mmole of maleic anhydride/5 ml of protein solution (43 moles of maleic anhydride added/mole of lysine). At this level of reagent all the free amino groups were modified, and less than 1 mole of hydroxyl-bound maleyl groups could be detected/mole of protein (Figure 2). Protected and unprotected antibody preparations were always treated with hydroxylamine after maleylation in order to ensure that no maleyl groups were hydroxyl bound. Following maleylation, no unreacted lysyl groups could be detected by dinitrophenylation of the maleylated protein after treatment with hydroxylamine, and thus it appeared that all amino groups had been maleylated.

Maleylation converts the positively charged ammonium groups into negatively charged maleamate groups. Loss of combining sites could be due either to loss of a necessary positive charge (actually conversion into a negative charge) in the site or to the steric interference of the added group or to a combination of both.

The loss of approximately 50% of the antibody combining sites with unprotected specifically purified

TABLE IV: Recovery of Binding Activity of Anti-R_p Antibodies (Preparation A) on Hydrolysis of Amino-Bound Maleyl Groups by Incubation in pH 3.5 Buffer at 37° for Various Times.

Sample	Incubation Time (hr)	Amino-Bound Maleyl Groups Removed[a] (%)	Combining Sites[b] (per mole of protein)	K_0[c] × 10^{-5} (l./mole)	a[d]
Unmodified	0		1.9	1.6	0.7
Unmodified	96		2.0	1.8	0.8
Modified[e]	0	0	1.0	0.2	0.7
Modified	48	69	1.9	0.7	0.8
Modified	72	80	2.0	1.0	0.8
Modified	96	88	2.0	1.3	0.8

[a] The maleyl groups were removed by dialysis in 0.1 M citric acid, 0.01 M phosphate, and 0.002 M EDTA buffer (pH 3.5) at 37°. [b] Values obtained by extrapolation of the binding curves as described in the Methods section. [c] Average binding constant. [d] Heterogeneity index. [e] Essentially all free amino groups were modified as determined by optical density measurements as well as by dinitrophenylation of the modified proteins as described in the Methods section.

anti-R_p antibodies (preparation A) following complete modification of essentially all amino groups is very clearly demonstrated in Figure 4 and Table I. The K_0 for this sample was reduced approximately eightfold. This suggests that the anti-R_p antibodies with higher binding constants may be more susceptible to maleylation than those with lower binding constants. The presence of 10^{-2} M *p*-nitrobenzenearsonate completely protected against the loss of any antibody combining sites during the maleylation procedure, and the K_0 of the protected sample was only slightly reduced (twofold) after maleylation. Thus the maleylation of groups outside the antibody site cannot be responsible for the lower K_0 value found for sites in the unprotected sample. In view of the conversion of some 70 positively charged amino groups into 70 negatively charged maleyl groups, and in view of the conformational changes reported by Habeeb *et al.* (1958) for the succinylation of bovine γ-globulin, it is interesting that possible conformational changes do not appear to have greatly affected the anti-R_p antibody sites in the maleylated preparations.

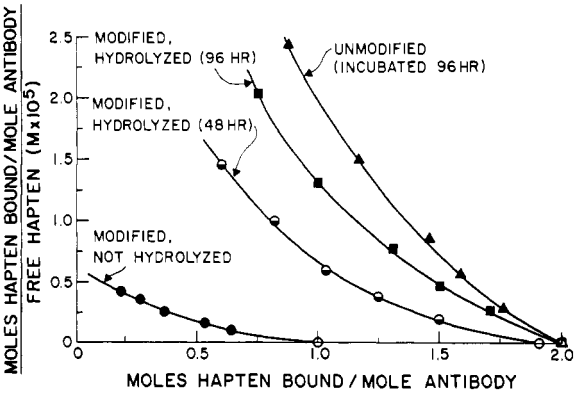

FIGURE 10: Binding curves showing the recovery of combining sites and restoration of the average binding constant when amino-bound maleyl groups are hydrolyzed from the maleylated anti-R_p antibodies (preparation A). The unmodified antibody preparation was exposed to the identical removal conditions for 96 hr at 37° with no loss of antibody activity. The maleylated antibody preparation demonstrated complete recovery of combining sites after 72-hr hydrolysis, and the average binding constant was restored to 72% of its original value after 96-hr hydrolysis.

That some conformational change did occur after maleylation of antibody molecules was demonstrated by the fact that the average sedimentation coefficient of the maleylated antibody samples was reduced from 6.6 (unmodified sample) to 6.3 S (modified sample). The nature of the conformational changes occurring after maleylation is presently under investigation.

The fact that the presence of hapten during maleylation completely prevented loss of antibody combining sites, and the absence of hapten during maleylation resulted in a loss of approximately 50% of the antibody combining sites provides a direct demonstration of the presence of an amino group (or groups) in a significant proportion of antibody sites directed against the negatively charged *p*-azobenzenearsonate hapten. The complete protection against loss of antibody sites by the presence of hapten during maleylation strongly suggests that the loss of combining sites was not due to any possible conformational changes caused by modification of amino groups outside of the antibody combining sites. The most direct interpretation of the hapten protection effect is that the hapten prevents modification of a residue in the site rather than prevents modification of a residue outside of the site whose modification could cause a conformational change affecting the site.

Antibodies prepared against the positively charged A_p hapten and antibodies prepared against the neutral P_3 hapten did not lose any antibody combining sites after maleylation, and the K_0 was practically unaltered in the unprotected, protected, and control samples (Figures 8 and 9 and Table III). These results demonstrate the absence of amino groups in the sites of antibody molecules prepared against these neutral and

positively charged haptens, since complete modification of amino groups was attained.

The absence of any effect on anti-A_p and anti-P_3 antibody combining sites strengthens the interpretation, based on the protection of anti-R_p antibodies by hapten during maleylation, that the loss of sites following modification of amino groups in anti-R_p antibodies is due to a direct modification of an amino group (or groups) in the sites of these antibodies. If the loss of antibody combining sites were simply due to modification of amino groups outside the combining site producing conformational changes affecting the combining site, one might expect that a similar loss of sites would be observed when all the amino groups in anti-A_p and anti-P_3 antibodies were modified. Such losses of antibody activity were not found with anti-A_p and anti-P_3 antibodies.

Other antihapten antibodies directed against negatively charged haptens showed evidence for direct involvement of amino groups in their combining sites. Unprotected anti-X_p and anti-P_p antibodies lost a significant number of combining sites after complete maleylation (Figures 6 and 7 and Table II). Unprotected anti-X_p antibodies lost 40% of their combining sites after maleylation, and unprotected anti-P_p antibodies lost 30% of their combining sites following modification of all their amino groups. In both antibody preparations the loss of combining sites could be protected by the presence of 10^{-1} M hapten during maleylation. The protection was more efficient with anti-P_p antibodies than with anti-X_p antibodies. The reduction in the average binding constant for these two protected antibody samples suggests that conformational changes have occurred. The inability to get good protection with anti-X_p antibodies has been observed previously (Chen *et al.*, 1962). It could result from the modification of amino groups at the periphery of the combining sites, which would affect the conformation of the combining sites and would be very difficult to protect against during the modification procedure because of the small size of the hapten used for protection.

Additional evidence for the existence of amino groups in a significant proportion of the combining sites of anti-R_p antibodies (preparation A) is shown by the recovery of activity on removal of maleyl groups (Figure 10 and Table IV) by hydrolysis in citrate–phosphate buffer (pH 3.5) at 37° (Figure 1). The maleylamino bond is very stable above pH 6 but is readily hydrolyzed below pH 5 by an intramolecular catalysis (Butler *et al.*, 1967). The intramolecular hydrolysis is catalyzed by the un-ionized carboxyl group which is held in a *cis* configuration by the unsaturated double bond in the maleyl group (Bender, 1957). Under these mild conditions, peptide bonds are not cleaved.

It appears that during maleylation the amino group (or groups) in the site is more resistant to modification since loss of sites resulted, for the most part, only when the final 10–20% of the free amino groups were modified (Figure 3). The reverse appears to be true for the recovery of sites after removal of the maleyl groups since 95% of the combining sites was recovered when more than 30% of the amino groups was still modified (Figure 10 and Table IV).

We are now in a position to make a comparison of the effect of maleylation and the effect of alanylation since we have now modified the same antibody preparation (anti-R_p antibody (preparation A)) by both techniques.

The unprotected anti-R_p antibodies lost 50% of their combining sites after maleylation, while the same unprotected anti-R_p antibody preparation lost only 25% of its antibody combining sites after alanylation.[5] This observation suggests that the larger number of combining sites lost after maleylation as compared with alanylation was due to the fact that the maleylated antibodies had more of their amino groups modified (100%), while the alanylated anti-R_p antibodies had only 65–70% of their amino groups modified. The anti-R_p antibody lost only 38% of its antibody activity when only 70% of the amino groups was modified by maleylation (Figure 3). It lost 73% of its antibody activity when 100% of its amino groups was maleylated.

The effect of maleylation differs from preparation to preparation since another anti-R_p antibody (preparation B) lost only 20% of its antibody combining sites (Figure 5 and Table I) when all of its amino groups were modified. Therefore, considerable differences exist in the proportion of combining sites having amino groups among antibody preparations, even when directed against the same hapten.

The important fact established by the present investigation is that populations of antibody sites directed against certain anionic haptens have been found in which, for each, some sites contain an amino group whereas other sites do not. Thus a chemical basis which can contribute to the observed binding heterogeneity of such populations of sites has been firmly established.

Whether or not anti-A_p and anti-P_3 preparations can ever be found which contain a detectable proportion of sites with amino groups remains a matter for speculation. In the case of antibody sites against the cationic A_p group, the presence of a positively charged ammonium group would seem to be highly unlikely.

Maleic anhydride was first used by Hartley as a reversible blocking agent for the selective and complete modification of amino groups. We have successfully employed maleic anhydride to demonstrate directly the presence of amino groups in a significant proportion of combining sites with antibodies prepared against certain negatively charged haptens. The reagent also demonstrated the absence of amino groups in the combining sites of antibodies prepared against a positively charged and a neutral hapten.

Acknowledgments

We thank Mr. F. Maenza and Miss B. Maren for their able technical assistance, Mr. R. Chrzanowski for the amino acid analyses, and Mr. L. Rendina for the preparation of ^{125}I-labeled haptens.

[5] Unreported observation.

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The Covalent Structure of a Human γG-Immunoglobulin. I. Isolation and Characterization of the Whole Molecule, the Polypeptide Chains, and the Tryptic Fragments*

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ABSTRACT: A human γG-immunoglobulin (Eu) has been purified from the plasma of a patient with multiple myeloma in order to provide material for amino acid sequence analysis of the entire molecule. Eu and its constituent heavy and light chains have been characterized by ultracentrifugal and immunological analyses, and their amino acid compositions and end groups have been determined. The molecular weight of the intact molecule (154,000) is consistent with the presence of two light chains (mol wt 23,500) and two heavy chains (mol wt 51,600). Amino acid analyses and end group determinations on Eu and the isolated chains also support this conclusion. Limited tryptic digestion of partially reduced and alkylated Eu produced fragments similar to the Fab and Fc fragments produced by papain digestion.

Structural studies have shown that immunoglobulins are multichain proteins (Edelman, 1959; Edelman and Poulik, 1961) made up of a four-chain unit containing two light and two heavy polypeptide chains (Fleischman *et al.*, 1962; Edelman and Benacerraf, 1962; Fougereau and Edelman, 1965). It is currently believed that the information for the specificity of antibodies resides in the amino acid sequences of light and heavy chains (Haber, 1964; Whitney and Tanford, 1965) and that the range of specificities is reflected in a large variety of sequences. Normal immunoglobulins are therefore heterogeneous, and it is difficult to carry out amino acid sequence analysis even on preparations of highly purified antibodies to a single hapten antigen. In the disease multiple myeloma, plasma cell tumors may be found which produce homogeneous immunoglobulins having the characteristic multichain structure (Edelman and Poulik, 1961; Edelman and Gally, 1962). Following the demonstration that urinary Bence–Jones proteins from patients with multiple myeloma are homogeneous light chains corresponding to those found in serum myeloma proteins (Edelman and Gally, 1962; Schwartz and Edelman, 1963), sequence studies have been carried out on

* From The Rockefeller University, New York, New York, and the Department of Biochemistry, Yale University, New Haven, Connecticut (W. H. K). *Received January 8, 1968.* This work was supported in part by grants from the National Science Foundation (GB-6546 and GB-6655) and the U. S. Public Health Service (AM 04256-08 and GM 12607-03). Part of this work was presented at the 32nd Cold Spring Harbor Symposium on Quantitative Biology, 1967 (Waxdal *et al.*, 1967); the Nobel Symposium 3 on γ-Globulins, Södergarn, Sweden, 1967 (Edelman, 1967); and the 7th International Congress of Biochemistry, Tokyo, 1967 (Edelman *et al.*, 1967). The nomenclature used is that recommended by the World Health Organization (1964).