Modification of Arginine in the Active Sites of Antibodies*

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ABSTRACT: Use of a 2,3-butanedione reagent to modify arginyl residues in proteins, as first described by Yankeelov *et al.* (Yankeelov, J., Jr., Kochert, M., Page, J., and Westphal, A. (1966), *Federation Proc. 25*, 590), has been applied to antibodies. Antibodies were acetylated to prevent any modification of lysine by the reagent. Modification of up to 70% of arginyl residues in antibodies against negatively charged haptens (*p*-azobenzoate, *p*-azobenzenearsonate, and succinamate) resulted in loss of active sites or in loss of specific binding activity. The presence of hapten during the modification reaction prevented such losses in whole or in part. Modification of arginine in antibodies against a positively charged hapten (*p*-azophenyltrimethylammonium) or a neutral hapten (3-azopyridine) resulted in no loss of sites or alteration of binding constants for these antibodies.

The results implicate the presence of arginine in the active sites of antibodies against the negatively charged haptens and the absence of arginine in the sites of antibodies against the positively charged and neutral haptens.

This report describes studies on the chemical modification of arginyl residues in antibody molecules under conditions mild enough to allow retention of antibody activity when the residues modified are other than those directly involved in antibody sites. The results provide a direct demonstration of the presence of arginine in the active site of antibodies against several negatively charged haptens and, concomitantly, the absence of arginine in the sites of antibodies against a positively charged and a neutral hapten.

Reagents for the chemical modification of arginyl residues in proteins have not been described until recently. Itano and Gottlieb (1963) and Toi *et al.* (1965, 1967) described the use of benzyl or 1,2-cyclohexanedione to modify arginyl residues in solutions of proteins containing 0.2 N or higher concentration of hydroxide ion. Few if any biologically active proteins can retain their activity after exposure to such highly alkaline conditions, even if no reagent is present. King (1966) has used malonaldehyde in 10 N HCl at 25° to convert arginine in proteins to δ-*N*-(2-pyrimidinyl)ornithine. Again, the biological activity of many proteins is irreversibly destroyed by 10 N HCl.

Yankeelov *et al.* (1966) have described the use of a reactive polymer of 2,3-butanedione to modify arginine under mild conditions (pH 8.2). We have used this reagent in the studies reported here. Recent information (J. Yankeelov, Jr., personal communication) indicates that the reactive polymer is probably the trimer.

Materials and Methods

Antigens. Azoproteins were prepared by coupling bovine γ-globulin (BGG)[1] with either *p*-diazoniumbenzoate, *p*-diazoniumbenzenearsonate, *p*-diazoniumbenzenesulfonate, 3-diazoniumpyridine, or *p*-diazoniumphenyltrimethylammonium; these antigens are designated, respectively, BGG-X_p, BGG-R_p, BGG-P_3, and BGG-A_p. The preparations have been described previously (Grossberg *et al.* (1962) and other references therein).

Succinamate groups were placed on BGG by succinylating the protein at pH 8 with 60 mg of succinic anhydride/g of protein, added in two equal portions at 10-min intervals to a 10% solution of the protein; the antigen is designated BGG-Su.

Antisera. Methods of preparing and testing rabbit antisera against *p*-azobenzoate (anti-X_p), *p*-azobenzenearsonate (anti-R_p), 3-azopyridine (anti-P_3), and *p*-azophenyltrimethylammonium (anti-A_p) have been described (Grossberg *et al.* (1962) and other references therein). Antisera against succinamate (anti-Su) were prepared by injecting rabbits in the footpads with 10 mg of BGG-Su in complete Freund's adjuvant plus weekly subcutaneous booster injections of 10 mg of antigen. Antisera were collected as above.

Antibody Preparations. Antibodies were specifically purified by either one of two methods. In the first method, used for purification of anti-X_p, anti-P_3, and anti-A_p, specific precipitates were prepared from decomplemented antiserum or the globulin fraction of antiserum by the addition of the optimal amount of specific antigen. The antigens were azo conjugates of the respec-

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[1] Abbreviations used: BGG, bovine γ-globulin; Su, succinate; anti-X_p, anti-R_p, anti-P_3, and anti-A_p, rabbit antisera against *p*-azobenzoate, *p*-azobenzenearsonate, 3-azopyridine, and *p*-azophenyltrimethylammonium, respectively.

tive haptens coupled to ovalbumin. Specific precipitates were washed and dissolved in 0.1–0.3 M solutions of the appropriate hapten, and the antibody separated from antigen by elution on a column of DEAE-cellulose with 0.01 M hapten solution at pH 7. Eluted antibody was precipitated with 20% (w/v) Na_2SO_4, dissolved, and extensively dialyzed against pH 8 borate-buffered saline to remove hapten. In the second method, used for purification of anti-R_p, antibody was absorbed on a solid immunoabsorbent and eluted with 1 M propionic acid in the cold, according to the methods described by Onoue *et al.* (1965) and Tanigaki *et al.* (1967). All specifically purified antibody preparations were more than 90% pure as determined by measurement of binding sites per mole of protein by the method of equilibrium dialysis. In the case of anti-Su, as well as some preparations of anti-X_p, the γ-globulin fraction of antiserum, prepared by the salt precipitation method of Kekwick (1940), was used.

^{125}I-Labeled Haptens. The preparation, by isotope exchange with ^{125}I carrier-free iodide, of ^{125}I-labeled *p*-iodobenzoate, *p*-iodobenzenearsonate, *p*-iodophenyltrimethylammonium, and 3-iodopyridine was by the same method that has been described previously (Grossberg *et al.*, 1962) for preparation of the ^{131}I-labeled haptens. ^{125}I-labeled *p*-iodosuccinanilate was prepared by succinylating ^{125}I-labeled *p*-iodoaniline with succinic anhydride. The exchange reaction to prepare the labeled amine was carried out at 108° for 1.5 hr in a sealed tube, utilizing the amine hydrochloride (10 μmoles) and 2.5 mc of ^{125}I in 0.3 ml of H_2O. The purified anilate had a specific activity of 100 mc/mmole.

Preparation of the 2,3-Butanedione Reagent. In preliminary experiments, the reagent was prepared by incubating 2,3-butanedione (reagent grade from Eastman Organic Chemicals) in 0.5 M phosphate buffer (pH 8.2) overnight at room temperature at a concentration of 15% (v/v), according to the method of Yankeelov *et al.* (1966). However, since studies with anti-R_p antibody were anticipated, we wished to avoid the presence of a high concentration of phosphate ion which is bound by the anti-R_p site. The use of either borate, Tris, or bicarbonate buffers was therefore investigated and it was found that a satisfactory reagent could be prepared in each of these buffers. Subsequent studies were standardized by the use of a reagent prepared in borate buffer as follows.

2,3-Butanedione (15 ml) is dissolved in about 80 ml of pH 8.2 borate buffer (20.55 g of H_3BO_3, 15.6 g of NaCl, and 2.13 g of NaOH per liter), the pH is adjusted to 8.7–8.9 with 5 N NaOH, and the final volume is made to 100 ml. After 6 hr at room temperature and again after 24 hr, the pH is readjusted to 8.8 with 1 N NaOH. The solution changes in color from yellow to red-brown and after 24–48 hr, the optical density at 400 mμ of a 15-fold dilution in borate buffer should be 0.45 or greater; the reagent is then usable without further adjustment of pH. If the value $OD^{1\%}_{400}$ 0.45 has not been reached, the pH is readjusted to 8.8 and the reagent allowed to stand an additional time. During subsequent standing in the dark, the reagent becomes darker colored, the pH falls to 7.5 or lower, and some precipitate appears. The reagent is usable for at least 10–14 days, during which time only the clear solution, obtained by decanting from the precipitate or by centrifuging, is used. Fresh reagent is made routinely every 10 days. The use of a higher initial pH in the preparation of the reagent, or readjustment of the pH after attaining the optical density noted, hastens precipitate formation.

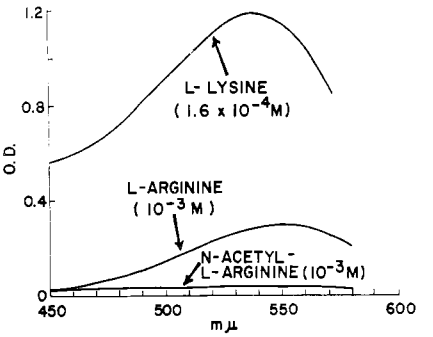

FIGURE 1: Absorption spectra of the reaction products of L-lysine, L-arginine, and *N*-acetyl-L-arginine with the diacetyl reagent. Reaction mixtures were read against a blank containing the equivalent amount of the diacetyl reagent only. Concentrations indicated are for the total amount of amino acid present in the solutions in both modified and unmodified form.

Amino Acid Analyses. Proteins were hydrolyzed in 6 N HCl (redistilled, constant boiling) in sealed ampoules flushed with N_2 for 20 hr at 110°. Analyses were performed on a Technicon Automatic amino acid analyzer, using a 6.5 hr "accelerated" gradient, with norleucine as internal standard.

Reaction of 2,3-Butanedione Reagent with Protein or Amino Acids. Preliminary studies using the reagent prepared in 0.5 M phosphate buffer revealed that both arginyl and lysyl residues of γ-globulin were modified. Furthermore, a red color (λ_{max} 530 mμ) which developed during incubation of γ-globulin with the reagent, and which was mentioned by Yankeelov *et al.* (1966) as being characteristic of the reaction of the reagent with guanidine hydrochloride or benzoylarginine methyl ester, or salmine, was found to be more related to reaction of the reagent with amino groups rather than with guanidinium groups in either γ-globulin or arginine. Thus, Figure 1 demonstrates the spectra of reaction mixtures of the reagent (1 ml) with lysine, arginine, or *N*-acetylarginine solutions (2 ml, containing 3×10^{-3} mmole of amino acid in 0.125 M phosphate buffer) after 24-hr incubation at room temperature. The figure demonstrates the intense red color produced in the mixture containing lysine (measured after diluting sixfold), the much less intense red color developed in the mixture containing arginine, and the complete absence of red color in the mixture containing *N*-acetylarginine. Thus, it appeared that monitoring optical density at 530 mμ would not give an indication of the extent of arginine modification in proteins, so that all subsequent deter-

TABLE I: Modification of Arginyl Residues in γ-Globulin (Protection of Lysyl Residues by Acetylation).

	OD_{530}[a]	Amino Acid Residues Recovd/100 Leucine Residues	
		Lysine	Arginine
γ-Globulin untreated		75	46
γ-Globulin + diacetyl reagent[b]	1.48	55	20
γ-Globulin, acetylated + diacetyl reagent[c]	−0.10[d]	75	19

[a] Mixtures were read against a blank in which 2 ml of 0.125 M pH 8.2 phosphate buffer replaced the protein solution; the readings were made at the end of the incubation period, before the mixtures were dialyzed. [b] Mixture of 2 ml of normal γ-globulin (3 mg/ml) in 0.125 M pH 8.2 phosphate buffer and 1 ml of diacetyl reagent (15%, v/v) in 0.5 M pH 8.2 phosphate buffer; incubated 6 hr at room temperature; dialyzed 18 hr against pH 8 saline. [c] Acetylated normal γ-globulin (2 ml) (3 mg/ml) in 0.125 M pH 8.2 phosphate buffer and 1 ml of diacetyl reagent (15%, v/v) in 0.5 M pH 8.2 phosphate buffer; incubated 18 hr at room temperature; dialyzed 18 hr against pH 8 saline. [d] Value at 6 hr was −0.08.

minations of extent of reaction with protein were done by amino acid analysis of acid hydrolysates.

The indication that lysine ε-amino groups as well as guanidinium groups could react with the reagent was established for γ-globulin by experiments such as that outlined in Table I, in which reaction of γ-globulin was compared with that of acetylated γ-globulin. Acetylation of γ-globulin was carried out with about 2 moles of acetic anhydride/mole of lysyl residue at pH 8 under conditions which have been previously shown to acetylate up to 90% of amino groups in γ-globulin (Grossberg and Pressman, 1963). The results of amino acid analyses indicated that approximately 55% of arginyl residues were modified in each instance. However, the acetylated γ-globulin gave no indication of modification of lysine residues whereas in the nonacetylated γ-globulin approximately 25% of the lysines were modified. The difference in the OD_{530} values for the two preparations also confirms that the red color appears to be due to lysine modification.

The Effect of the 2,3-Butanedione Reagent on the Charge of γ-Globulin. The effect of modification with the reagent on the charge of γ-globulin was investigated by determining the change in mobility of the γ-globulin. This was done by modifying both acetylated γ-globulin and γ-globulin amidinated with ethyl acetimidate under conditions (1 M reagent at pH 8.6 for 2 hr at 0°) which have been shown to amidinate about 85% of lysine ε-amino groups (Wofsy and Singer, 1963). Both types of preparation, following modification of 55–60% of their arginine, migrated more rapidly toward the anode, when subjected to immunoelectrophoresis at pH 8.4, than did the preparations before treatment with the reagent. The result was more clearly seen with the amidinated preparation whose mobility before butanedione treatment did not differ from that of original unmodified γ-globulin. Amino acid analyses confirmed the amidination of lysine residues, as well as the absence of modification of lysine by the reagent in both the amidinated and acetylated proteins. Thus the reagent removes some of the positive charge from groups other than lysine in γ-globulin at pH 8.4. There is a possibility that the charge of amidinium groups was affected by the reagent. Although the reduction of charge was observed at pH 8.4 with protein, titration of the reaction product of *N*-acetylarginine and the reagent indicated a pK_a of about 9.7 for the modified guanidinium group of *N*-acetylarginine.

Modification of Arginine in Antibodies in the Presence or Absence of Hapten. Subsequent treatment of antibodies with the reagent was routinely carried out with preparations acetylated as indicated above. The procedure was to mix one volume of protein solution with one volume of pH 8 borate buffer containing either no additional substances (unprotected sample) or containing 0.2–0.3 M hapten (protected sample). One volume of the reagent was then added to each sample. The mixtures were adjusted to pH 8.6 with 1 N NaOH (0.05–0.1 volume) and allowed to stand at room temperature for the desired time (1–24 hr). A control mixture in which the reagent was replaced by buffer was also prepared. The reaction was stopped after the shorter time intervals (up to 6 hr) by addition of 0.2 volume of 1 M phosphate buffer, pH 4, which brought the solutions to a pH below 6.5. Hapten was added to a final concentration of 0.1 M in the unprotected and control samples and the mixtures were then dialyzed first against pH 6 phosphate-buffered saline to remove most of the reagent and then exhaustively against several changes of pH 8 borate-buffered saline to remove hapten as well as the last portions of the reagent.[2] Mixtures to be treated more than 2 hr were maintained between pH 8.2 and 8.6 by addition of 1 N NaOH at 2-hr intervals up to 6 hr. Reaction

[2] In preliminary experiments, it was established that a decreased recovery of tyrosine in amino acid analysis of γ-globulin resulted when a small portion of the reagent was added to the protein before acid hydrolysis. Therefore dialysis of samples was continued until reagent was removed, as judged by absence of detectable color in a sample of the reagent, originally at 5% (v/v) concentration and containing no protein, which was dialyzed in parallel with the reaction mixtures. Dialysis for 36–48 hr against two or three changes of 1000 volumes of pH 8 borate-buffered saline usually was sufficient. Modified proteins, in which the reagent was removed according to this criterion, showed 95–100% recovery of tyrosine. Modified proteins, after dialysis, had a slight brown color which appeared to be due to modified arginyl residues and not due to residual reagent.

TABLE II: Amino Acid Analyses[a] of the Acetylated γ-Globulin Fraction of Anti-X_p Serum before (C) and after Reaction with Diacetyl Reagent (6 hr) in the Absence (A) or Presence (P) of 0.1 M *p*-Nitrobenzoate.

	Sample		
Amino Acid	C	A	P
Asp	109	108	109
Thr	162	163	166
Ser	133	135	134
Glu	126	128	127
Pro	113	115	114
Gly	111	112	112
Ala	72	75	72
Val	121	121	121
Cys (1/2)	44	44	44
Met	10.1	10.8	10.7
Ile	44	44	44
Leu	(90)	(90)	(90)
Tyr	54	52	52
Phe	44	43	44
Lys	68	68	68
His	17.1	17.8	17.3
Arg	**44**	**16.8**	**16.3**

[a] Values are residues recovered per mole of γ-globulin, assuming a value of 90 for Leu. Values for Thr and Ser are uncorrected for losses during hydrolysis.

mixtures brought to pH 8.6 after 6 hr were found to be at pH 8.2 ± 0.2 after a further 18-hr standing. Mixtures treated for 18–24 hr were dialyzed directly against pH 8 borate-buffered saline.

Binding of Haptens by Antibodies. The binding of ^{125}I-labeled haptens by antibodies was determined by the method of equilibrium dialysis, utilizing several free-hapten concentrations, as previously described (Grossberg and Pressman, 1960). The use of the Sips equation and the associated methods of plotting to analyze the data to obtain values for average binding constant (K_0), antibody site concentration (A_0), and heterogeneity index (a) have been described (Nisonoff and Pressman, 1958). Antibody activity, when expressed as "per cent binding activity," is defined as the concentration of hapten bound by an antibody preparation at a given free hapten concentration, divided by the concentration of hapten bound by a control antibody preparation at the same free hapten concentration, the quotient being multiplied by 100. The number of antibody sites per mole of protein in specifically purified antibody preparations was determined by extrapolation of binding data plotted according to the method of Scatchard (1949), as first utilized by Karush (1956).

Determination of Protein. The value of OD_{280} for 1 mg/ml of γ-globulin or antibody solutions was taken to be 1.46. Protein concentration of chemically modified

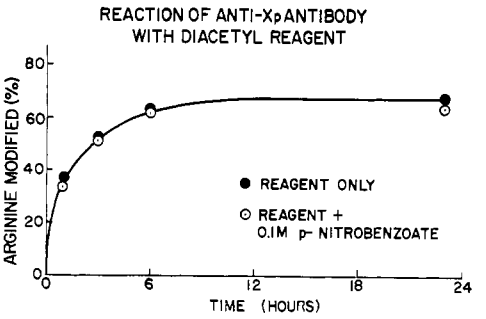

FIGURE 2: The extent of modification by diacetyl reagent of arginyl residues in the acetylated γ-globulin fraction of anti-X_p serum, in the presence and absence of 0.1 M *p*-nitrobenzoate as a function of time.

γ-globulin preparations was determined from the nitrogen content as measured after digestion and Nesslerization, assuming 16% nitrogen in the protein. The molecular weight of γ-globulin and antibodies was taken to be 160,000.

Results

Rate of Arginine Modification and Effect on Anti-X_p Activity Produced by the 2,3-Butanedione Reagent in the Presence and Absence of Hapten. The procedure for modifying arginyl residues as described in the Methods section above was applied to an acetylated γ-globulin fraction of anti-X_p serum at a final protein concentration of 20 mg/ml. One reaction mixture contained no hapten and a second mixture contained 0.1 M *p*-nitrobenzoate. Portions of the two mixtures were removed and brought to pH 6 after 1, 3, 6, and 23 hr of reaction. After dialysis part of each portion was subjected to amino acid analysis and the remainder was assayed for binding activity toward ^{125}I-labeled *p*-iodobenzoate. Results were compared with the amino acid analysis and binding activity of a portion of the γ-globulin fraction (control) which was not treated with the reagent but was otherwise carried through the same procedures. The data for this experiment are given in Figures 2 and 3 and Table II.

The amino acid analyses in Table II are representative of all the analyses in this experiment, and of many others carried out in the course of this investigation, in that arginine is seen to be the only amino acid residue whose amount is reduced following treatment of acetylated γ-globulin with the reagent and subsequent hydrolysis. No ninhydrin-reactive product, corresponding to a hydrolysis product of modified arginine, was detected. The extent of arginine modification was not affected by the presence of 0.1 M *p*-nitrobenzoate.

The rate of arginine modification is shown in Figure 2, in which the results of amino acid analyses for arginine in the reaction mixtures after 1, 3, 6, and 23 hr are plotted as per cent of the arginine in the control. Under the conditions of this experiment 63% of the arginine residues were modified after 6 hr, both in the presence

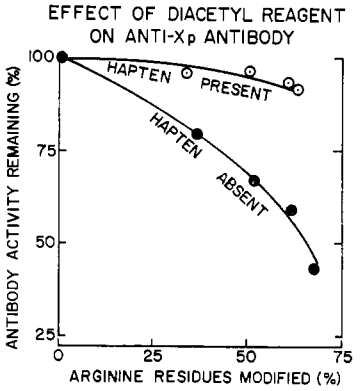

FIGURE 3: Curves showing loss of anti-X_p activity with increasing extent of modification of arginyl residues in the protein. Loss of anti-X_p activity is prevented when modification of arginyl residues is carried out in the presence of 0.1 M *p*-nitrobenzoate. Each point represents the mean of duplicate determinations of antibody activity, with an average deviation from the mean of 1%. Per cent activity (defined in the text) was determined at a free concentration of 3.12×10^{-6} M ^{125}I-labeled *p*-iodobenzoate; the γ-globulin fraction contained 14.5% antibody, with an average binding constant of 7.2×10^4 l./mole. Per cent activity of 100 corresponds to 5.78×10^{-6} M *p*-iodobenzoate bound at a total protein concentration of 12.8 mg/ml. The samples were portions of those whose arginine content is represented in Figure 2.

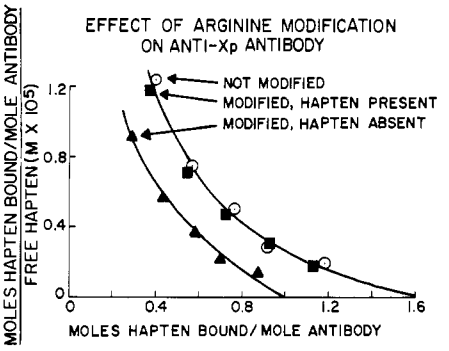

FIGURE 4: Binding curves showing the loss of sites due to modification of 54% of the arginyl residues in acetylated, specifically purified anti-X_p antibody in the absence of hapten. No sites are lost when the same extent of modification is carried out in the presence of 0.1 M *p*-nitrobenzoate. Each point represents the mean of duplicate determinations with an average deviation from the mean of ±1.2%.

and absence of hapten, and only about 5% additional residues were modified after 23 hr.

The effect of arginine modification on anti-X_p binding activity is shown in Figure 3. These data reveal a marked distinction between the mixture treated in the absence of hapten, in which there was a progressive loss in binding activity, so that only 43% of the original activity remained after 68% of the arginine was modified, and the mixture treated in the presence of hapten, in which over 92% of the binding activity remained after 63% of the arginine was modified. Thus the presence of hapten prevented loss of anti-X_p activity although the over-all extent of arginine modification was similar to that produced in the absence of hapten. This finding is strong evidence that arginyl residues in the active site of anti-X_p antibodies were modified by the diacetyl reagent.

Modification of Arginine in Specifically Purified Anti-X_p and Anti-R_p Antibody in the Presence or Absence of Hapten. The Effect on Antibody Sites. In order to examine the effect on antibody sites caused by modification of arginine, a preparation of acetylated specifically purified anti-X_p antibody was modified in the presence (protected sample) and absence (unprotected sample) of 0.1 M *p*-nitrobenzoate. Two separate experiments were performed with the anti-X_p preparation, using two different preparations of the reagent. In the first experiment, the reaction proceeded for 6 hr and the subsequent amino acid analyses indicated that 54% of the arginine was modified in both the protected and unprotected samples, compared to the control. Binding curves for these samples are given in Figure 4. Approximately 0.6 site/mole of antibody was lost in the unprotected sample, whereas essentially no sites were lost in the protected sample. In the second experiment, in which 24-hr treatment of the antibody resulted in modification of 71% of the arginine in both protected and unprotected samples, approximately 0.8 site/mole was lost in the unprotected, and only 0.2 site in the protected.

Treatment of specifically purified acetylated anti-R_p antibody with the reagent was carried out in the presence and absence of 0.1 M *p*-nitrobenzenearsonate, re-

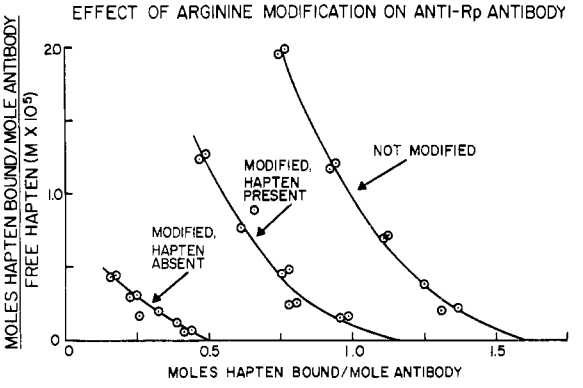

FIGURE 5: Binding curves showing the extent of loss of sites due to modification of 54% of the arginyl residues in acetylated specifically purified anti-R_p antibody in the presence and in the absence of hapten. Fewer sites are lost when the modification is carried out in the presence of 0.1 M *p*-nitrobenzenearsonate. Each point represents an individual determination.

TABLE III: Loss of Binding Sites and Effect on Binding Constants Following Modification of Arginine in Specifically Purified Anti-X_p and Anti-R_p Antibodies.

Sample	Arginine Content (% of control)	Sites/Mole of Protein	$K_0 \times 10^{-5}$ (l./mole)	a[a]
Acetylated anti-X_p				
Control	100	1.7	0.5	0.65
Protected[b] (0.1 M *p*-nitrobenzoate)	46	1.7	0.5	0.65
Unprotected	46	1.1	0.7	0.70
Acetylated anti-X_p				
Control	100	1.7	0.5	0.65
Protected (0.1 M *p*-nitrobenzoate)	29	1.5	0.5	0.65
Unprotected	29	0.95	0.6	0.70
Acetylated anti-R_p[b]				
Control	100	1.6[c]	2.5	0.8
Protected (0.1 M *p*-nitrobenzene-arsonate)	46	1.15	1.5	0.7
Unprotected	46	0.45	1.5	0.7
Anti-R_p				
Control	100	2.05	4.0	1.0
Protected[d] (0.1 M *p*-nitrobenzene-arsonate)	45	1.07	3.0	1.0
Unprotected[d]	43	0.50	3.2	1.0

[a] Heterogeneity index. [b] >95% of NH_2 groups acetylated as determined by amino acid analysis following treatment with excess fluorodinitrobenzene according to the method of Porter and Sanger (1948). [c] The lower number of sites of this acetylated antibody preparation compared to the unacetylated preparation (next below) probably reflects loss of sites involving lysyl residues (see Chen *et al.*, 1962). [d] Lysine content of these samples was 82–85% of control.

sulting in the modification of 54% of the arginine in both the protected and unprotected samples compared to the control. The binding curves for these samples (Figure 5) indicate loss of 1.1 sites/mole when hapten was absent, and only 0.4 site/mole when hapten was present.

Another portion of the same anti-R_p preparation was treated with diacetyl reagent without prior acetylation; 71% of the arginine was modified in both the protected and unprotected samples. The binding data for these samples are summarized in Table III (bottom). Table III also contains the values of binding constants and heterogeneity indices calculated for the other samples of specifically purified anti-R_p and anti-X_p studied. The data in Table III confirm the previous evidence (Figure 3) that arginine is present in the site of anti-X_p antibodies, and extend this finding to the sites of anti-R_p antibodies. There was no marked change in the average binding constants of sites remaining in the various antibody preparations following modification.

Evidence for the Presence of Arginine in the Sites of Antisuccinamate Antibodies. A preliminary survey was made of the effect of the reagent on the γ-globulin fraction of anti-Su antiserum. Loss of antibody sites accompanied modification of arginine. This loss was protected against by hapten as shown by the data in Table IV.

Absence of Arginine in the Sites of Anti-A_p and Anti-P_3 Antibodies. When 70% of the arginine in specifically purified acetylated anti-A_p antibody was modified, the binding curve of the resultant preparation was indistinguishable from that of the unmodified preparation

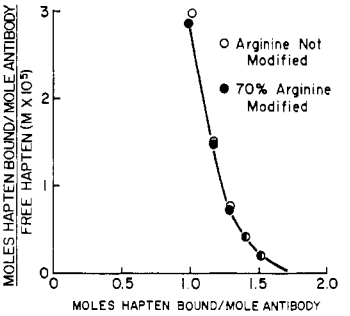

FIGURE 6: Binding curves showing no loss of sites or any change in their average binding constant following modification of 70% of the arginyl residues in acetylated specifically purified anti-A_p antibody. Each point represents the mean of duplicate determinations with an average deviation from the mean of ±0.8%.

TABLE IV: Loss of Binding Sites and Effect on Binding Constant Following Modification of Arginine in the γ-Globulin Fraction of Antisuccinamate Antiserum.

Sample	Arginine Content (% of control)	Binding Site Concn[a] (% of control)	$K_0 \times 10^{-5}$ (l./mole)	a^b
Control	100	100	2.4	0.5
Protected (0.08 M succinanilate)	45	90	1.7	0.5
Unprotected	45	75	1.5	0.5

[a] Values obtained by extrapolation of binding curves. [b] Heterogeneity index.

TABLE V: Absence of Effect on Binding Sites and Binding Constants Following Modification of Arginine in Specifically Purified Anti-P_3 Antibody.

Sample	Arginine Content (% of control)	Sites/Mole of Protein	$K_0 \times 10^{-5}$ (l./mole)	a^a
Acetylated anti-P_3				
Control	100	1.60	0.85	1.0
Protected (0.1 M pyridine)	27	1.53	0.90	1.0
Unprotected	28	1.53	0.90	1.0
Anti-P_3				
Control	100	1.85	0.70	1.0
Protected[b] (0.1 M pyridine)	25	1.78	0.80	1.0
Unprotected[b]	26	1.74	0.75	1.0

[a] Heterogeneity index. [b] Lysine content of these samples was 88% of control.

(Figure 6). An analogous result was obtained with a specifically purified anti-P_3 preparation in which 73% of the arginine was modified (Table V). The absence of any effect of arginine modification on either binding sites or average binding constants is evident for both of these antibody preparations.

Discussion

The loss of binding sites of specifically purified anti-X_p and anti-R_p antibodies following modification by the 2,3-butanedione reagent and the protection by hapten against such loss are very clearly demonstrated by the binding curves in Figures 4 and 5. Loss of activity of antibody against a third negative hapten (antisuccinyl antibody) is also documented in Table IV.

On the other hand, antibodies against the positively charged *p*-azophenyltrimethylammonium hapten and against the uncharged 3-azopyridine group are unaffected by the reagent as shown by the data in Figure 7 and Table V. Moreover, the findings with these antibodies against haptens which are not negatively charged demonstrate that essentially no alteration in the configuration of the antibody sites results from the modification of even 30 arginyl residues in the antibody molecule, since the binding constants of sites in the modified antibodies are not different from those of unaltered antibodies. The absence of any effect on anti-A_p and anti-P_3 sites due to modification of so many arginines in these molecules strengthens the interpretation, based on the protection by hapten, that the loss of sites or activity following modification of arginine in anti-X_p, anti-R_p, and anti-Su antibodies is due to an attack in the sites of these antibodies and not due to an alternate mechanism, *i.e.*, a change in their sites following modification elsewhere in the molecule. Furthermore, the observations render unlikely the possibility that the protective effect of hapten is due to a conformation change caused by hapten, which "buries" a previously reactive residue (removed from the site) whose modification causes the observed losses of activity.

It would appear from the results here that arginine is present in antibody sites directed against the negatively charged haptens. Antibody sites against anionic haptens have previously been shown to contain a positive charge required to provide much of the binding energy for specific interaction with the negatively charged haptens (Pressman, 1958; Pressman *et al.*, 1961a). The amino

acid residues contributing the positive charge in the majority of sites in preparations of both anti-X_p and anti-R_p antibodies have been shown to be other than lysine, although a small proportion of sites containing lysine have been detected in some anti-R_p antibody preparations (Chen *et al.*, 1962). Such a residue could be arginine or histidine. No evidence has been found for the presence of histidine in the site of anti-X_p antibodies (Pressman *et al.*, 1961b) although this does not rule out its presence. If histidine is being modified in the present work it would have to be regenerated completely during the acid hydrolysis prior to amino acid analysis.

The possibility that the loss of activity is due to the modification of an uncharged or negatively charged amino acid which is subsequently regenerated appears unlikely, particularly in view of the differences between the behavior of antibodies against negative groups and the behavior of those against other groups.

Thus the interpretation we favor is that it is the modification of arginine in the sites of antibodies against negatively charged haptens which is responsible for the observed effect of the 2,3-butanedione reagent to cause loss of sites in these antibodies. The lack of effect of the reagent on antibodies against a positively charged or a neutral hapten is interpreted as being due to the absence of arginine in these antibody sites.

The results of this investigation indicate that 2,3-butanedione reagent should prove of use in examining the role of arginine in the active sites of other biologically active proteins, since it is capable of modifying arginyl residues under mild conditions which do not destroy activity unless an arginyl residue is important.

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