ACKNOWLEDGMENTS

The authors wish to express their indebtedness to Drs. G. E. Hein, J. B. Jones, and R. A. Wallace for providing experimental data prior to its publication and to Dr. G. E. Hein for assistance in the preparation of this communication.

REFERENCES

Abrash, H. I., Kurtz, A. N., and Niemann, C. (1960), *Biochim. Biophys. Acta 45*, 378.
Applewhite, T. H., Martin, R. B., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1457.
Applewhite, T. H., and Niemann, C. (1959), *J. Am. Chem. Soc. 81*, 2208.
Braunholtz, J. T., Kerr, R. J., and Niemann, C. (1959), *J. Am. Chem. Soc. 81*, 2852.
Brenner, M., and Huber, W. (1953), *Helv. Chim. Acta 36*, 1109.
Brown, H. C. (1938), *J. Am. Chem. Soc. 60*, 1325.
Dixon, M., and Webb, E. C. (1958), Enzymes, New York, Academic Press, Inc., pp. 172-179.
Gordon, T. P. (1959), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.
Hein, G. E., and Niemann, C. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1341.
Hein, G. E., and Niemann, C. (1962), *J. Am. Chem. Soc.* in press.
Herbst, R. M., and Shemin, D. (1943), *Org. Syn.*, Coll. Vol. II, p. 11.
Huang, H. T., and Niemann, C. (1952), *J. Am. Chem. Soc. 74*, 4634.
Huang, H. T., and Niemann, C. (1953), *J. Am. Chem. Soc. 75*, 1395.
Jones, J. B., and Niemann, C. (1962), *Biochemistry 1*, 1093.
Lutwack, R., Mower, H. F., and Niemann, C. (1957), *J. Am. Chem. Soc. 79*, 5690.
Meyer, H., and Graf, R. (1928), *Ber. 61*, 2202.
Nielands, J. B., and Cannon, M. D. (1955), *Anal. Chem. 27*, 29.
Northrop, J. H. (1961), *Ann. Rev. Biochem. 30*, 1.
Peterson, P. E., Wolf, J. P., III, and Niemann, C. (1958), *J. Org. Chem. 23*, 303.
Ramberg, L. (1907), *Ber. 40*, 2588.
Rothstein, B. (1932), *Bull. Soc. Chim. 51*, 838.
Waite, H., and Niemann, C. (1962), *Biochemistry 1*, 250.
Wolf, J. P., III (1959), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Effect of Acetylation on the Active Site of Several Antihapten Antibodies: Further Evidence for the Presence of Tyrosine in Each Site*

A. L. GROSSBERG AND D. PRESSMAN

From the Department of Biochemistry Research, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, New York

Received July 26, 1962

Extensive modification of four antihapten antibodies with acetic anhydride resulted in loss of binding activity in each case. The presence of hapten during acetylation partially prevented these losses. This observation indicated that a group in the site of each antibody was being attacked. Reversal of the losses of binding activity by treatment of the acetylated antibodies with hydroxylamine indicated that a hydroxyl group had been acetylated in each site. In view of this fact and other data previously reported, it was concluded that a tyrosine residue is present in each of the antibody sites. Of the antibodies studied (anti-*p*-azobenzenearsonate [anti-R_p], anti-*p*-azobenzoate [anti-X_p], anti-*p*-azophenyltrimethylammonium [anti-A_p], and anti-3-azopyridine [anti-P_3]) only anti-R_p and anti-X_p appeared to lose activity by partial acetylation of amino groups in the molecule. Of these, only the former appeared to have an amino group in the site. Acetylation of mixtures of two antibodies and analysis of complete binding curves of modified antibodies allowed determination of the relative sensitivity of the antibodies to acetylation and of the relative effects of acetylation on blocking binding sites and on alteration of binding constants. It could be concluded that the importance for binding of the acetylatable group varied for the several antibodies. The sensitivity toward acetylation was in the order, anti-$R_p \geq$ anti-$X_p \gg$ anti-$A_p >$ anti-P_3.

We have been studying the nature of antibody sites by determining the effect of chemical modification on specific binding activity. Whether the effect is due to reaction of a group in the binding site itself rather than elsewhere in the molecule can be determined by carrying out the alteration in the presence and in the absence of the specific hapten. If the hapten protects against the loss of sites which is caused by alteration in the absence of hapten, then the loss must have been due to reaction of a group in the site.

Recently we compared four different antihapten antibodies with respect to the effect of iodination on binding activity (Grossberg *et al.*, 1962). We studied antibodies to haptens bearing a positive charge, a negative charge, and no charge, to give a broad range of different antibody types. The antibodies studied were anti-*p*-azobenzoate (anti - X_p), anti - *p* - azobenzenearsonate (anti - R_p), anti - *p* - azophenyltrimethylammonium (anti-A_p), and anti - 3 - azopyridine (anti - P_3). Iodination of each of these was found to reduce antibody activity, and it was concluded that each of the antibodies contained an iodinatable residue, probably tyrosine, in the antibody site.

Nisonoff and Pressman (1959) reported that extensive acetylation of anti-X_p antibody with acetic anhydride resulted in loss of antibody sites. We are now reporting the effect of acetylation with acetic anhydride on the antibody activity of all four of the above antihapten antibodies. Antibody activity is lost when each anti-

* Supported by Grant No. E-2342 from the National Institute of Allergy and Infectious Diseases. Presented in part at the 138th Meeting of the American Chemical Society, New York, New York, September, 1960, and at the 45th Annual Meeting of the Federation of American Societies for Experimental Biology (*Fed. Proc. 20*, 108, 1961), Atlantic City, N. J., March, 1961.

body is acetylated, and in each instance this loss is partially prevented when hapten is present during acetylation. Furthermore, the effects of acetylation can be largely reversed in each instance if the acetylated antibody is treated with hydroxylamine. From these results the conclusion may be drawn that a hydroxyamino acid residue in the site of each antibody is acetylated under the conditions employed. The hydroxyl group involved is most probably that on the tyrosine residue previously implicated by the iodination study cited above.

The effects of acetylation have been analyzed in terms of the proportion of antibody sites affected and the binding constants of sites remaining. The relative sensitivity of the several antibodies toward acetylation has also been investigated.

Materials and Methods

Methods of preparing and testing each of the antisera studied have been described (Grossberg *et al.*, 1962, and other references therein). γ-Globulin fractions of antisera were prepared by the method of Kekwick (1940) and utilized as the antibody-containing protein. The γ-globulin fraction of normal rabbit serum was similarly prepared. These fractions were 95 to 98% γ-globulin as determined by free boundary electrophoresis. The contaminant was a slowly migrating β-globulin.

The I^{131}-labeled haptens, *p*-iodobenzoate (Nisonoff and Pressman, 1958), *p*-iodophenyltrimethylammonium (Grossberg and Pressman, 1960), and *p*-iodobenzenearsonate and 3-iodopyridine (Grossberg *et al.*, 1962), were prepared by isotope exchange as previously reported.

Acetylation of γ-Globulins.—γ-Globulin solutions (40 to 45 mg/ml) were acetylated at 5° in a recording *p*H-stat in which the *p*H was maintained at 8.0 or 6.0 by addition of 1 M or 4 M NaOH and a record kept of the base consumed. Small amounts (1 to 5 mg) of acetic anhydride were added as the ice cold aqueous solution. Larger amounts of acetic anhydride were added in 10 or 20 mg portions, the reaction of each portion being allowed to proceed to completion (5 to 10 minutes) before addition of the next portion. The maximum amount of reagent added was that which resulted in the first appearance of precipitate due to salting out of protein by sodium acetate formed (*ca.* 1.5 M). The acetylated proteins were dialyzed against a large volume of *p*H 8 borate-buffered saline. All were completely soluble after dialysis.

Treatment of Proteins with Hydroxylamine.—γ-Globulin or acetylated γ-globulin solutions at *p*H 9.5 were made 8.0 M with respect to hydroxylamine, the *p*H was adjusted to 9.5 over the next 10 to 15 minutes if necessary, and the mixture was kept at 5° for 18 to 20 hours. The hydroxylamine was removed by dialysis at *p*H 8.

Binding of Haptens.—The binding of I^{131}-labeled haptens by the various modified and unreacted antibodies was measured by the method of equilibrium dialysis at *p*H 8, as previously described (Grossberg and Pressman, 1960). All determinations were in duplicate and were corrected for the nonspecific binding of hapten by normal γ-globulin or acetylated normal γ-globulin. The *p*H of samples containing I^{131}-labeled 3-iodopyridine was lowered to 3 or less by addition of HCl after their removal from the dialysis bags after equilibration, in order to prevent volatilization of this compound during counting. In the case of binding by a mixture of two antibodies, separate portions of the mixture were equilibrated against each hapten involved. Samples to be compared at a given hapten concentration were dialyzed against a common hapten solution so that the free hapten concentration at equilibrium was identical for all.

Analytical Methods.—Protein concentration was determined by nitrogen analysis, 16.0% being used as the nitrogen content of γ-globulin. Some unmodified proteins were determined by measurement of optical density at 280 mμ ($E_{1cm}^{1\%}$ = 14.6). Free amino groups in protein were determined by the method of Peters and Van Slyke (1932).

Results

Acetylation of Antibodies in the Presence and Absence of Hapten.—Preliminary experiments indicated that acetylation decreased the binding of hapten by anti-R_p, anti-A_p, and anti-P_3 antibodies in a manner similar to that previously reported for the effect on anti-X_p binding (Nisonoff and Pressman, 1959). In three separate experiments, the effect of extensive treatment of antibodies with acetic anhydride was studied in the presence and absence of hapten. Moreover, a direct comparison of the effects of acetylation on two antibodies at a time was made by paired reactions in which two antibodies were mixed prior to acetylation so that they were exposed to identical conditions. Anti-R_p globulin, mixtures of anti-R_p and anti-A_p globulins, and mixtures of anti-A_p and anti-P_3 globulins were treated with an appropriate amount of acetic anhydride in the presence and in the absence of added hapten. After dialysis of the treated materials, portions of all were assayed for free amino content and the remainder utilized for hapten binding measurements (Table I). In all treated samples a high degree of acetylation of amino groups was achieved irrespective of whether hapten was present.

With each of the antibodies, acetylation decreased the ability to bind hapten. When the corresponding antibodies were acetylated in the presence of 0.1 M hapten they retained ability to bind hapten to varying extents (Table I). In the presence of heterologous hapten, anti-R_p retained only 23% of its original activity after acetylation, but retained 51% of its activity when acetylated in the presence of 0.1 M homologous hapten. More extensive exposure of a mixture of anti-R_p and anti-A_p to acetic anhydride resulted in no retention of anti-R_p activity and only 16% retention of anti-A_p activity, whereas exposure to acetic anhydride in the presence of a mixture of the R_p and A_p haptens (0.1 M each) resulted in some retention of anti-R_p activity (about 8%) and 43% retention of anti-A_p activity.[1] Acetylation of the mixture of anti-A_p and anti-P_3 with half the amount of acetic anhydride in the presence and absence of hapten resulted in only 23% retention of anti-A_p activity in the absence of hapten, but over 50% retention when hapten was present. The corresponding retention of anti-P_3 activity was 19% and 27%. Thus, the protection by 0.1 M hapten of anti-R_p and anti-A_p sites was clearly demonstrable. The pro-

[1] With regard to the protection of anti-R_p antibody by *p*-nitrobenzenearsonate, we have observed that when this hapten is added to unmodified antibody and the mixture then dialyzed exhaustively to remove the hapten, binding activity of the anti-R_p antibody is not fully recovered. Whether this is due to the presence of some particularly strong binding antibody or to some undialyzable hapten contaminant has not been determined. This effect has not been observed in the other antibody systems studied. It is noteworthy that in some experiments (*e.g.* Table I, footnote *d*) *p*-nitrobenzenearsonate was added to a mixture of anti-R_p and anti-A_p, but only reduction of anti-R_p activity was observed, showing that the effect is a specific one.

TABLE I

EFFECT OF ACETYLATION IN THE PRESENCE AND ABSENCE OF HAPTEN ON ANTIHAPTEN ANTIBODIES

Antibody	Exposure (Mole Acetic Anhydride/ Mole Protein)	Degree of Amino Acetylation (%) Without Hapten	Degree of Amino Acetylation (%) With Hapten[a]	Protein Concentration (mg/ml)	Free Hapten[b] (M × 10⁶)	Hapten Bound by: Unmodified Antibody	Hapten Bound by: Acetylated Antibody	Hapten Bound by: Antibody Acetylated in Presence of Hapten (M × 10⁶)[c]	Normal γ-Globulin	Acetylated Normal γ-Globulin
Anti-R_p	1 × 120	73[d]	75	16.0	6.39	5.57 **100%**	1.25 **23%**	2.81 **51%**	0.10	0.02
Anti-R_p + Anti-A_p	10 × 250	92	100	20.0	4.01	4.26[e] **100%**	0.04 **1%**	0.33 **8%**	0.09	0.01
				20.0	4.33	8.73[e] **100%**	1.40 **16%**	3.72 **43%**	0.37	0.70
Anti-A_p + Anti-P_3	5 × 250	95[f]	91	20.0	6.12	7.67 **100%**	1.76 **23%**	3.94 **52%**	0.39	0.74
				19.0	5.96	4.16 **100%**	0.80 **19%**	1.11 **27%**	0.50	1.07

[a] Modification of anti-R_p was in the presence of 0.1 M *p*-nitrobenzenearsonate; anti-R_p + anti-A_p, in 0.1 M *p*-nitrobenzenearsonate, and 0.1 M *p*-iodophenyltrimethylammonium; anti-A_p + anti-P_3, in 0.1 M *p*-iodophenyltrimethylammonium and 0.1 M pyridine. [b] Haptens were the homologous I^{131}-labeled *p*-iodo derivatives (see Materials). [c] Average of duplicate determinations; deviation from the mean was less than 0.07 except where noted below in *e*. Values for antibody are corrected for binding by normal γ-globulin or acetylated normal γ-globulin; figures in bold type are per cents of the binding shown by unmodified antibody. [d] 0.1 M *p*-iodobenzoate was present in this sample. [e] These values (with deviations from the mean of ±0.16 and ±0.01 respectively) were obtained after addition of 0.1 M each of *p*-nitrobenzenearsonate and *p*-iodophenyltrimethylammonium to the antibody mixture and then dialyzing exhaustively as with the sample acetylated in the presence of the hapten mixture. The binding by a sample of the same anti-R_p–anti-A_p mixture which was not exposed to hapten was, for anti-R_p, 4.97×10^{-6} M; for anti-A_p, 8.73×10^{-6} M. [f] 0.1 M each of the *p*-iodophenyltrimethylammonium and pyridine were added to this sample after acetylation and the sample was then exhaustively dialyzed.

tection of the anti-P_3 site was less marked, but detectable.

Comparison of the Sensitivity of anti-R_p, anti-X_p, and anti-A_p Antibodies to Acetylation.—The data of Table I show differences in the effectiveness of acetylation in reducing the binding activity of the various antibodies. Hence mixtures of anti-R_p and anti-A_p antibodies and of anti-R_p and anti-X_p antibodies were acetylated to several different extents and the hapten binding activities and degree of amino acetylation were measured. The acetylations of the anti-R_p–anti-A_p mixture were carried out at *p*H 8 at four levels of acetic anhydride exposure, molar ratios of anhydride to protein of approximately 30, 120, 1000, and 2500 to 1 being used. The acetylations of the anti-R_p–anti-X_p mixture were carried out at *p*H 8 and *p*H 6, at the first three of the four levels mentioned above. The results of these experiments are plotted in Figures 1 and 2. It may be seen that anti-R_p and anti-X_p antibodies (Fig. 1) lost activity progressively with increasing acetylation (as measured by loss of free amino groups on the protein); the former antibody seemed somewhat more sensitive. There was little difference between the effects observed after acetylations at *p*H 6 and 8, except that exposure at the lower *p*H was less effective in acetylating amino groups. In contrast, anti-A_p was much less sensitive than anti-R_p to acetylation (Fig. 2). At a level of amino acetylation at which over 60% of anti-R_p activity was lost, only a few per cent loss of anti-A_p activity was detected. Only at the highest levels of acetylation, at which anti-R_p activity was completely destroyed, were losses of anti-A_p activity apparent.

Effect of Acetylation on Combining Sites and on Hapten Binding Constants.—The fact that protection by hapten against the loss of activity following acetylation was observed in the case of anti-R_p, anti-A_p, and anti-P_3 antibodies constitutes evidence that in each antibody a group in the site was attacked. However, part of the effect of acetylation could be due to a decrease in the average binding constant (K_0) of the sites rather

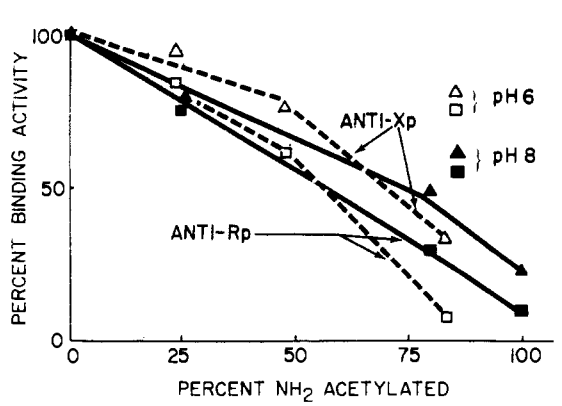

FIG. 1.—Effect of acetylation at different levels on hapten binding by a mixture of anti-R_p and anti-X_p antibodies. Binding of haptens is expressed as per cent of the binding by the unacetylated mixture.

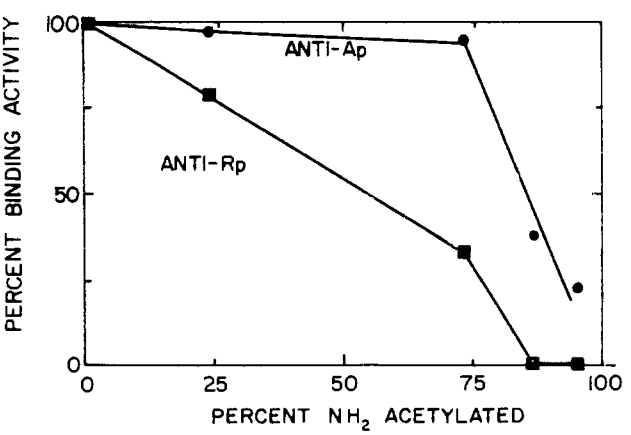

FIG. 2.—Effect of acetylation at different levels on hapten binding by a mixture of anti-R_p and anti-A_p antibodies. Binding of haptens is expressed as per cent of the binding by the unacetylated mixture.

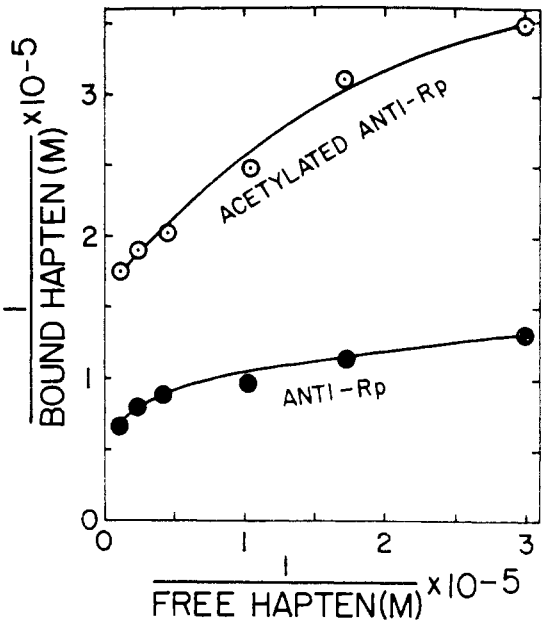

FIG. 3.—Binding of I^{131}-labeled *p*-iodobenzenearsonate by anti-R_p preparations (17.0 mg protein per ml): for lower curve, $A_0 = 1.76 \times 10^{-5}$ M; for upper curve $A_0 = 0.77 \times 10^{-5}$ M. Correction for binding by normal γ-globulin, $(n_0) = 4.0 \pm 0.7\%$ of free hapten concentration; by acetylated normal γ-globulin, $(n_a) = 0.0 \pm 0.5\%$.

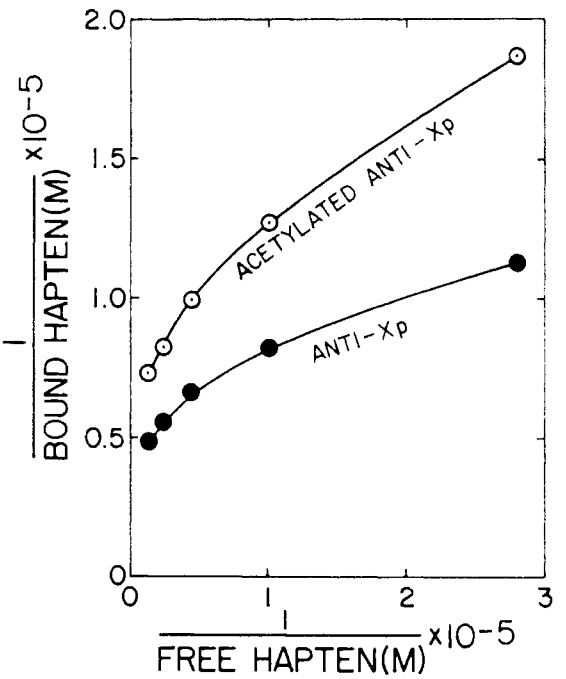

FIG. 4.—Binding of I^{131}-labeled *p*-iodobenzoate by anti-X_p preparations (20.0 mg protein per ml): for lower curve, $A_0 = 3.8 \times 10^{-5}$ M; for upper curve, $A_0 = 2.5 \times 10^{-5}$ M. $n_0 = 6.8 \pm 0.9\%$; $n_a = 0.5 \pm 0.2\%$ (see legend for Fig. 3).

than complete blocking of sites. In order to assess how much loss of binding activity was due to reduction of K_0 and how much was due to complete loss of site, binding of hapten by acetylated antibodies and by the corresponding unmodified preparations was determined at several free hapten concentrations. Binding by anti-A_p and anti-P_3 antibodies acetylated (as a mixture) in the presence of hapten was also measured. In addition, acetylation of a different preparation of anti-

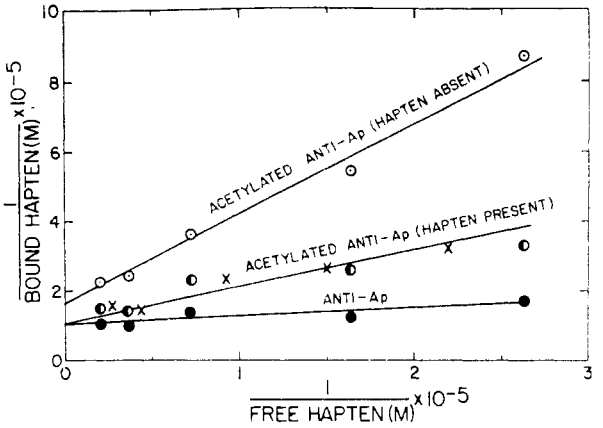

FIG. 5.—Binding of I^{131}-labeled *p*-iodophenyltrimethylammonium by a mixture of 167 mg anti-A_p and 250 mg anti-P_3 preparations (19.4 mg protein per ml): for lower curve, $A_0 = 1.00 \times 10^{-5}$ M; for upper curve, $A_0 = 0.66 \times 10^{-5}$ M; ×, same values as ◐, but plotted with heterogeneity index = 0.8 (abscissa values are $\times 10^{-4}$), $A_0 = 1.00 \times 10^{-5}$ M. $n_0 = 6.9 \pm 0.5\%$; $n_a = 13.5 \pm 1.0\%$ (see legend for Fig. 3).

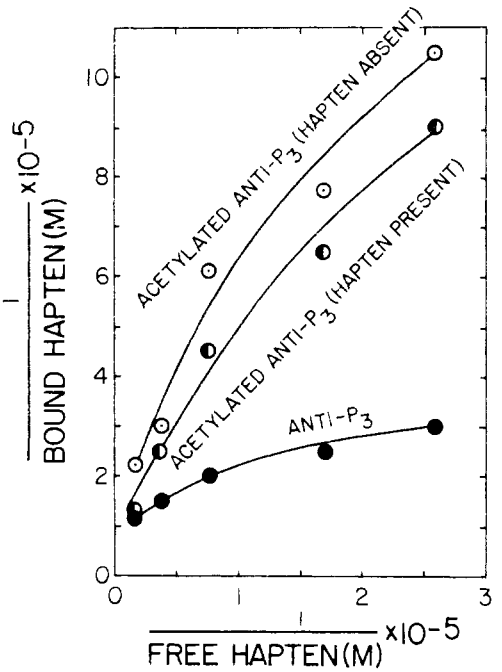

FIG. 6.—Binding of I^{131}-labeled 3-iodopyridine by a mixture of anti-A_p and anti-P_3 preparations (same samples as in Fig. 5); A_0 in each case $= 1.4 \times 10^{-5}$ M. $n_0 = 7.5 \pm 0.5\%$; $n_a = 11.0 \pm 1.5\%$ (see legend for Fig. 3).

P_3 antibody was carried out at a higher level of exposure to acetic anhydride in both the presence and absence of hapten.

The binding data are plotted as the reciprocal of bound hapten concentration against the reciprocal of free hapten concentration (Figs. 3, 4, 5, 6, and 7). The extrapolation of such curves to infinite free hapten concentration, i.e., the ordinate intercept, provides a measure of the total concentration of binding sites. In the instances in which the data were fitted by a curve, intercepts were obtained by transforming the plots to straight lines by use of the Sips equation (Sips, 1948) as described previously (Grossberg *et al.*, 1962).

The values of total site concentration (A_0), average combining constant (K_0), and heterogeneity index (a)

TABLE II

EFFECT OF ACETYLATION OF ANTI-HAPTEN ANTIBODIES ON TOTAL BINDING SITES (A_0) AND AVERAGE COMBINING CONSTANTS (K_0)

	(1) Anti-R_p	(2) Anti-X_p	(3) Anti-A_p[a]	(4) Anti-P_3[a]	(5) Anti-P_3
Antibody content of γ-globulin[b] (mg/mg total protein)	0.083	0.15₂	0.075	0.120	0.107
Exposure (moles Ac_2O/mole protein)	1 × 120	1 × 120	5 × 250	5 × 250	4 × 500
Amino groups acetylated (% of original)					
In absence of hapten	75	80	95	95	97
In presence of hapten[c]	—	—	91	91	95
Binding sites remaining after acetylation (% of original)[b,d]					
In absence of hapten	44	66	61	100	71
In presence of hapten[c]	—	—	100	100	100
Average binding constants (liters/mole × 10^{-5})[b,d]					
Before treatment	1.6	0.21	4.0	0.40	0.91
After acetylation in absence of hapten	1.1	0.21	0.65	0.13	0.014
After acetylation in presence of hapten[c]	—	—	0.87	0.15	0.029
Heterogeneity index (a)[b]					
Before treatment	0.50	0.45	1.0	0.60	0.60
After acetylation in absence of hapten	0.50	0.50	1.0	0.80	0.70
After acetylation in presence of hapten[c]	—	—	0.80	0.80	0.60

[a] These two preparations were treated in a mixture (see text). [b] Values were obtained from the extrapolated binding curves (Figs. 3, 4, 5, 6, and 7). [c] Haptens employed (each at 0.1 M) were: for anti-A_p, *p*-iodophenyltrimethylammonium; for anti-P_3, pyridine. [d] Haptens employed for binding were the homologous *p*-iodo derivatives, labeled with I^{131}.

thus obtained (Table II) reveal that acetylation of the more sensitive anti-R_p and anti-X_p antibodies can result in the loss of binding sites with little or no change in binding constant of the remaining sites. More intensive acetylation of the less sensitive anti-A_p antibodies caused loss of sites, accompanied by a decrease of binding constant to about one sixth its initial value. Acetylation of anti-A_p antibody in the presence of hapten resulted in essentially complete retention of sites, but reduction of their binding constant to about one fifth the initial value. Acetylation of one anti-P_3 antibody preparation (done in the same experiment with anti-A_p by acetylating a mixture of the two)

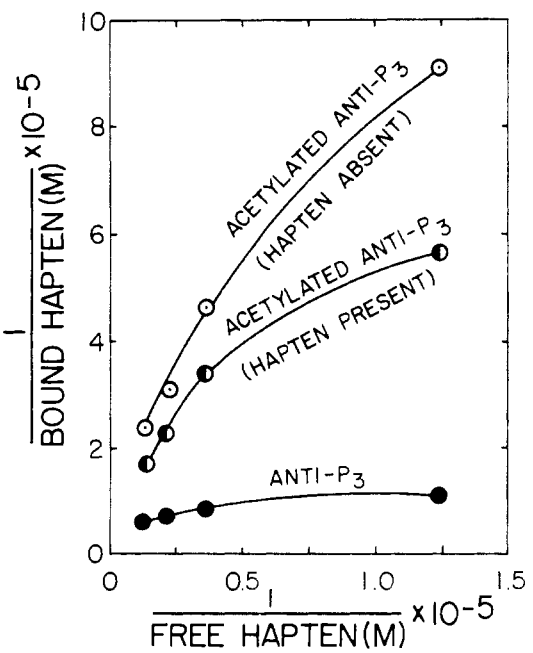

FIG. 7.—Binding of I^{131}-labeled 3-iodopyridine by anti-P_3 preparations (15.0 mg protein per ml); for lower curve, $A_0 = 2.0 \times 10^{-5}$ M; for upper curve, $A_0 = 1.43 \times 10^{-5}$ M; for middle curve, $A_0 = 2.0 \times 10^{-5}$ M. Corrections for nonspecific binding as in Figure 6.

resulted in no loss of binding sites, but a decrease of K_0 to one third its initial value. The presence of hapten during acetylation of this anti-P_3 antibody afforded no protection against alteration of the binding constant (K_0 was the same as when the antibody was acetylated with hapten present).

Another anti-P_3 preparation, when acetylated at a still higher level of exposure in the absence of hapten, lost about 30% of its active sites. This loss of sites was completely prevented when acetylation was performed in the presence of hapten (Fig. 7; Table II). Acetylation of this preparation also resulted in a large decrease in the average binding constant of remaining sites (to about one sixtieth of the original value). Hapten prevented about half of this decrease (Table II). It is noteworthy that this anti-P_3 preparation was the same one utilized in the previously reported study of the effect of iodination on anti-P_3 (Grossberg *et al.*, 1962; Fig. 3).

Reversal of Acetylation Effect by Hydroxylamine.—Hydroxylamine is known to split acetyl esters with formation of free alcohol and acet-hydroxamic acid. It has been shown by Uraki *et al.* (1957) that acetylation of protein hydroxyl groups can be demonstrated by this means and that acetylated amino groups in protein are apparently not split by the reagent. Thus it would be expected that if antibody sites were inactivated by acetylation of hydroxyl groups the inactivation might be reversed by hydroxylamine, whereas if the inactivation were due to amino acetylation reversal would not occur. That the former possibility is the case for all the antibodies studied here is borne out by the data in Table III. Various acetylated antibody preparations, some from experiments recorded above, were treated with hydroxylamine. The binding activity of these materials after removal of hydroxylamine was compared to that of portions of the corresponding acetylated materials and to that of the unmodified antibodies, previously treated with hydroxylamine or not. Each set of samples was equilibrated against a common volume of hapten solution to allow direct comparison of the binding activities. The results (Table III) are expressed as per cent binding activity compared to that of the unmodified protein. With

TABLE III
REVERSAL OF ACETYLATION BY HYDROXYLAMINE FOR ANTIHAPTEN ANTIBODIES

Antibody	% Binding Activity of Acetylated Samples[a,b]: Prior to Hydrolysis with Hydroxylamine	% Binding Activity of Acetylated Samples[a,b]: After Hydrolysis with Hydroxylamine[c]	Preparation Used	Acetic Anhydride Applied (Moles per Mole Protein)[d]	Hapten Concentration: Free (M × 10^5)	Hapten Concentration: Bound by Unacetylated Sample[b] (M × 10^5)
Anti-R_p	**31**	**60**	[e]	1 × 120	0.64	0.57
	10	**57**	Fig. 1	5 × 200[f]	0.62	0.60
	8	**42**	Fig. 1	5 × 200	0.62	0.60
	1	**35**	Fig. 2	10 × 250	0.57	0.60
Anti-X_p	**60**	**83**	[e]	1 × 120	0.57	1.53
	35	**67**	Fig. 1	5 × 200[f]	0.48	0.28
	25	**56**	Fig. 1	5 × 200	0.48	0.28
Anti-A_p	**27**	**73**	Fig. 2	10 × 250	0.69	0.91
Anti-P_3	**71**	**82**		1 × 120[g]	0.51	1.32
	20	**50**		5 × 200	0.66	1.21

[a] Calculated as $\frac{\text{Hapten bound by acetylated sample}}{\text{Hapten bound by unacetylated sample}} \times 100$. [b] Values are average of duplicates (average deviation ±1.3%), determined at a protein concentration of 20 mg/ml, except where noted. [c] 0.3 M hydroxylamine; *p*H 9.5; 18 hours at 5°. [d] Acetylation at *p*H 8. Except where noted, acetic anhydride was added in replicate portions as indicated. [e] These samples were run as a mixture; protein concentration for binding measurements = 32 mg/ml. [f] Acetylation at *p*H 6. [g] Free amino groups per mole in this preparation: before acetylation, 63 ± 2; after acetylation, 20.1 ± 1.5; after acetylation and hydroxylamine treatment, 21.7 ± 1.5.

every set of samples there was a marked increase in the binding of acetylated antibody after treatment with hydroxylamine. Hydroxylamine treatment had little or no effect on binding by the unmodified antibodies. One set of samples was analyzed, and no detectable gain in free amino groups was found after hydroxylamine treatment of the acetylated protein. This indicated that acetamino groups were not hydrolyzed.

DISCUSSION

The results reported here show that extensive acetylation of four antihapten antibodies produces loss of ability of each to bind the corresponding hapten. Since each antibody can be protected by the specific hapten from this loss of activity during acetylation (Table I), it appears that a group in the site of each is attacked by acetic anhydride.[2] Demonstration of loss of sites by acetylation, and their partial retention when acetylation is performed in the presence of hapten, is also afforded by the binding curves (Figs. 3, 4, 5, and 7 and Table II).

The nature of the amino acid residue in each site, the acetylation of which could account for these results, is not directly apparent. Acetic anhydride can attack protein amino groups (Putnam, 1953) and aliphatic and phenolic hydroxyl groups (Uraki *et al.*, 1957). "Exposed" amino groups are apparently quite reactive and are acetylated early in the course of modification. The fact that some loss of anti-R_p binding activity is observed when a relatively moderate percentage of protein amino groups are acetylated (Figs. 1 and 2) suggests that the amino group may be implicated in the site of this antibody. This interpretation is supported by the observation (Chen *et al.*, 1962) that carbamylation of amino groups in anti-R_p results in loss of sites, and this loss can be protected against by hapten.

There is some loss of activity of anti-X_p antibody as amino groups are acetylated, but to a lesser extent than with anti-R_p antibody (Fig. 1). This might indicate some contribution of an amino group to this site. However, carbamylation (Chen *et al.*, 1962) indicated that there was no amino group in the site. Although there was loss of binding activity, it appeared to be due to attack elsewhere than in the site because clear-cut protection of the site was not afforded by hapten. The loss of activity following moderate acetylation would thus appear to be due either to attack of an amino elsewhere than in the immediate site or to acetylation of a hydroxyl in the site.

A considerable body of evidence has been accumulated which indicates that a positive charge is involved in the sites of antibodies directed against anionic haptens (Pressman, 1958; Pressman *et al.*, 1961a). However, this charge may be contributed by a guanidinium group rather than by an ammonium group.

The lack of any effect of acetylation on binding by anti-A_p antibody until most of the amino groups are reacted (Fig. 2) makes it appear very unlikely that an amino group is involved in this site unless it is exceptionally unreactive. In addition, the fact that this site is directed toward a cationic hapten provides a theoretical basis for considering the presence of a cationic group in this site unlikely.

There is considerable evidence for the participation of a hydroxyamino acid in the site of each of the four antibodies studied. The fact that the four antibodies can be protected by their specific haptens from loss of activity or of sites due to extensive acetylation (Tables I and II, and Nisonoff and Pressman, 1959) indicates that a residue in the specific site of each antibody is attacked by acetic anhydride. In addition, the fact that the loss of activity by acetylation can be reversed by hydroxylamine (Table III) indicates that a hydroxyl group in the sites is being attacked. The fact that each of the four antibodies contains a group in its site which can be iodinated has been reported previously (Grossberg *et al.*, 1962). Anti-X_p antibody has also been found (Pressman *et al.*, 1961b) to have an ionizable group in its site with a *pK* of about 10.

[2] There is the possibility that the observed protection of site by hapten takes place by a less direct mechanism. Thus acetylation of a particular residue elsewhere than in the site might produce a conformational change in the site so that combination cannot take place. Then if a conformational change takes place when hapten is bound to the site so that this particular residue is rendered less reactive toward acetylation, a specific protection effect would be observed.

The only amino acid residue which can be iodinated and acetylated and which has an ionizable group at *p*H 10 is tyrosine. It is possible of course that more than one hydroxyamino acid is involved in the observed effects. Thus a site containing both tyrosine and serine or threonine would be expected to be modified in the observed manner as well as a site containing only tyrosine.

Analysis of the effects of acetylation in terms of proportion of sites lost and effect on binding constants (Table II) gives information on the involvement of the acetylatable group in the sites. The group which is acetylated in the sites of the several antibodies is involved to different extents in the specific interaction of site with hapten. With some antibodies, *i.e.*, anti-R_p and anti-X_p, acetylation of the group in the site appears to destroy binding activity completely, whereas acetylation elsewhere than in the site does not affect the activity of the site (*i.e.*, sites on such antibodies have the same binding constant as those on unmodified antibody). These sites must also be unaffected by any distortion due to acetylation in other parts of the molecule which might occur due to the radical change in charge distribution (the protein as a whole suffered a net increase in negative charge of 40 to 50 units), or due to the steric effects of introduction of acetyl groups.

The anti-A_p site is less readily affected by acetic anhydride. The group in this site which can be acetylated appears to be important for specific interaction, although less reactive with the reagent than the group acetylated in anti-R_p and anti-X_p sites, since anti-A_p sites are lost when sufficient acetylation of the molecule has occurred. This extent of modification also decreases the binding constant of remaining sites appreciably. It appears that alterations in other parts of the molecule produced by the more intensive exposure to acetic anhydride may be sufficient to distort the anti-A_p site. Habeeb *et al.* (1958) have observed that a high degree of acetylation, and more notably of succinylation, causes expansion of protein molecules. The fact that the change in K_0 was observed with anti-A_p and not with anti-X_p and anti-R_p is probably due to the more intensive exposure to acetic anhydride required to destroy binding activity of anti-A_p. It is probable that equally intensive exposure of anti-X_p or anti-R_p would result in some change of K_0 for the few remaining sites. Thus varying degrees of chemical modification of a group in the antibody site might in general be expected to reduce K_0 to values ranging from the original constant to zero (complete blocking of site). Under the condition of our experiments, hapten binding by sites having an average binding constant less than $^1/_{100}$ of that of the unmodified site would not be detected, since nonspecific binding of hapten becomes too great a factor (see legends for Figures 3, 4, 5, 6, and 7).

The anti-P_3 site is relatively resistant to alteration by acetylation (similar to anti-A_p). A hydroxyamino acid (probably tyrosine) is involved in the anti-P_3 site in some manner, as evidenced by hydroxylamine reversal of acetylation effects (Table III) and hapten protection of the site against iodination (Grossberg *et al.*, 1962). The anti-P_3 site is apparently somewhat less affected by acetylation than the anti-A_p site (Table II, columns 3 and 4). This may be due either to a lesser reactivity of the acetylatable group in the anti-P_3 site or to a lesser importance of this group for binding. More intensive exposure of the anti-P_3 molecule to acetic anhydride (Table II, column 5) leads to detectable loss of sites. At the same time there is an even greater effect on K_0 (compare columns 4 and 5, Table II), which indicates that the anti-P_3 site is sensitive to an alteration which seems to have little to do with over-all charge effects (since amino acetylation was essentially complete in both instances). Most probably, hydroxyl acetylation accounts for the effect. Thus, if tyrosine is involved, it may be peripheral to the anti-P_3 site, so that its chemical modification greatly reduces the strength with which hapten is bound to the site but does not prevent detectable binding at a sufficiently high hapten concentration. It might be supposed, further, that this tyrosine is so situated with respect to the site that the presence of hapten in the site does not prevent acetylation of the hydroxyl group as much as it hinders introduction of an iodine; this would account for the marked protection of the site against iodination previously observed (Grossberg *et al.*, 1962). (This last reference also discusses other possible causes of incomplete protection of sites by hapten.)

In all the above remarks it has been tacitly assumed that only one kind of site is involved for each antibody. However, the nonlinear binding curves give evidence of heterogeneity in every case. Thus an alternate interpretation of many of the above-mentioned observations is possible. For example, the proportionately greater reduction in binding in the region of lower free hapten concentration, noted especially in the case of acetylated anti-P_3 (Fig. 7), may be due not to a reduction in average binding constant of all sites but to destruction of a small number of sites having a high binding constant. These sites could be considered to contain a tyrosine hydroxyl which plays a critical role in binding hapten, whereas the remaining sites may be of a different structure and not involve tyrosine to an important extent or at all. Since extrapolation of binding curves with precision greater than $\pm$ 10% of total sites is difficult, loss of binding by up to 20% of total sites might easily remain undetected in the extrapolation.

Acknowledgments

We wish to thank Mr. Leonard Rendina and Mr. Gerald Radzimski for technical assistance.

References

Chen, C. C., Grossberg, A. L., and Pressman, D. (1962), *Biochemistry 1*, 1025.

Grossberg, A. L., and Pressman, D. (1960), *J. Am. Chem. Soc. 82*, 5478.

Grossberg, A. L., Radzimski, G., and Pressman, D. (1962), *Biochemistry 1*, 391.

Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 587.

Kekwick, R. A. (1940), *Biochem. J. 34*, 1248.

Nisonoff, A., and Pressman, D. (1958), *J. Immunol. 80*, 417.

Nisonoff, A., and Pressman, D. (1959), *J. Immunol. 83*, 138.

Peters, J. P. and Van Slyke, D. D. (1932), Quantitative Clinical Chemistry, Vol. II, Baltimore, Md., The Williams and Wilkins Co., pp. 386–399.

Pressman, D. (1958), *in* Serological and Biochemical Comparison of Proteins, Cole, W. H., editor, New Brunswick, N. J., Rutgers Univ. Press, p. 32.

Pressman, D., Nisonoff, A., and Radzimski, G. (1961a), *J. Immunol. 86*, 35.

Pressman, D., Nisonoff, A., Radzimski, G., and Shaw, A. (1961b), *J. Immunol. 86*, 489.

Putnam, F. W. (1953), *in* The Proteins, Vol. 1, Part B, Neurath, H., and Bailey, K., editors, New York, Academic Press, Inc., p. 925.

Sips, R. (1948), *J. Chem. Phys. 16*, 490.

Uraki, Z., Terminiello, L., Bier, M., and Nord, F. F. (1957), *Arch. Biochem. Biophys. 69*, 644.