Ia was pyrolyzed at 100°. After 2 hours of heating, 0.02 g. (7.41%) of methyl acetate was collected. After 5 hours of heating a total of 0.03 g. (11.1%) of ester was obtained.

Preparation of N,N-Diphenylaminoethyl Acetate.—Following the procedure of Paul,[16] 18.8 g. (0.088 mole) of N,N-diphenylaminoethanol[17] and 10.2 g. (0.1 mole) of acetic anhydride were refluxed for 2 hours. The reaction mixture was then diluted with water and the *p*H of the solution adjusted to 9 with sodium hydroxide solution. The alkaline solution was extracted with ether, the combined extracts washed with water and dried over sodium sulfate. Removal of the ether followed by distillation afforded 15.1 g. (67.4%) of N,N-diphenylaminoethyl acetate, b.p. 174.5–175° at 3 mm., sp. gr. 20/20 1.1120, $n^{22.5}$D 1.5821; *MR* calcd. 74.60, found 74.31; reported[16] b.p. 140–145° at 1–2 mm.

Anal. Calcd. for $C_{16}H_{17}NO_2$: C, 75.27; H, 6.71; N, 5.49. Found: C, 75.40; H, 6.34; N, 5.51.

Pyrolysis of N,N-Diphenylaminoethyl Acetate. A. At 250°.—A 5.1-g. sample of N,N-diphenylaminoethyl acetate was heated, under a nitrogen atmosphere, at 250° for 2 hours. No volatile products were collected.

B. At 500°.—A 8.9-g. (0.036 mole) sample of the ester was passed through a Vycor tube packed with 1/8 inch helices and heated to 500° by a furnace. The ester was passed through the tube at a rate of 1.5 g. per minute. The pyrolysate, collected in a Dry Ice trap, was extracted with water and the aqueous solution diluted to 100 ml. A 10-ml. aliquot, titrated with 0.0111 *N* sodium hydroxide solution using a Beckman model G *p*H meter, consumed 19.8 ml. of base and indicated that 0.132 g. (6.1%) of acetic acid was produced. The water-insoluble, brown oil from the pyrolysate was chromatographed on an alumina column but no N,N-diphenylvinylamine could be isolated.

Pyrolysis of 2-(N-β-Hydroxyethylanilino)-4,6-dimethoxy-*s*-triazine (VII). Run No. 1.—A 3.0-g. (0.01 mole) sample of the dimethoxy-*s*-triazine alcohol was pyrolyzed at 200–260° for 1.5 hours. No volatile products were observed. The residue in the distillation flask, after recrystallization from dioxane, afforded 1.56 g. (65%) of 2-N-vinylanilino-4-hydroxy-6-methoxy-*s*-triazine (IIa), m.p. 254–256°. A mixture melting point with a sample of IIa from pyrolysis of Ia was not depressed.

Run No. 2.—A 20.0-g. (0.07 mole) sample of VII was pyrolyzed under the same conditions. The distillate, 1.0 g. (43.8%), was identified as methanol, b.p. (cor.) 63.3°, n^{20}D 1.3296; reported b.p. 64.6°,[18a] $n^{20.5}$D 1.3288.[18b]

(16) P. T. Paul, U. S. Patent 2,401,658, June 4, 1946.

(17) Generously supplied by the Naugatuck Chemical Co.

(18) (a) W. R. G. Atkins and T. A. Wallace, *J. Chem. Soc.*, **103**, 1469 (1913); (b) F. H. Getman and V. L. Gibbons, THIS JOURNAL, **37**, 1995 (1915).

[CONTRIBUTION FROM DEPT. OF BIOCHEMISTRY RESEARCH, ROSWELL PARK MEMORIAL INSTITUTE, BUFFALO, NEW YORK]

Nature of the Combining Site of Antibody against a Hapten Bearing a Positive Charge[1]

BY ALLAN L. GROSSBERG AND DAVID PRESSMAN

RECEIVED MARCH 3, 1960

Previous work indicated that the specific site of antibody against the positively charged phenyltrimethylammonium ion contains a negative charge. The present report emphasizes this and shows that the negative charge is apparently a carboxylate group since esterification of the antibody carboxyl groups by diazoacetamide destroys binding activity. This destruction is not due to the esterification which occurs elsewhere than in the specific site. Moreover esterification of antibody to a negative hapten does not affect its ability to bind hapten.

One approach to the exact composition of the antibody site, *e.g.*, which chemical groups contribute to the intimate structure of the site, is to modify the site chemically by use of reagents for reactive groups in protein. This approach has been used with varying success.[2–8] Reagents which react with only one type of residue are few[9,10] and reaction conditions must be mild enough to permit retention of biological activity. Care must be taken to assure that reaction in other parts of the large antibody molecule does not obscure effects on the site or confuse the interpretation of data. Thus acetylation of antihapten[5–7] or antiprotein[3,4,7] antibodies has been shown to reduce or prevent precipitation by antigen but this is due mainly to reaction with amino groups in regions elsewhere than in the combining site.[6] Specific protection of the site by hapten during treatment with reagent provides a means of proving that the destructive reaction is in the site.[2] It was by such methods that acetylation, when extensive, was shown to affect the combining site.[6]

The use of antihapten sera has a distinct advantage over the use of antiprotein sera in such studies in that information concerning the combining site of antibodies is more easily interpreted in the former case. This is because, unlike antibodies against complex antigens such as proteins, the antihapten antibodies are directed against a known chemical configuration. Such systems have also been of value in indicating the presence of a charged group in the antibody site; *i.e.*, a positively charged group is in the site homologous for the negatively charged *p*-(*p*-azobenzene)-azobenzoate (X_p)[11] and 4-azophthalate[12]; also a negative charge is in the site homologous for the positively charged *p*-azophenyltrimethylammonium ion (A_p).[13] Measurement of hapten binding by dialysis equilibrium allows detection of interaction at the site without the com-

(1) Presented at the 136th National Meeting of the American Chemical Society, Atlantic City, Sept. 1959. Supported in part by a grant from the National Institute of Allergy and Infectious Diseases, USPHS.

(2) D. Pressman and L. A. Sternberger, *J. Immunol.*, **66**, 609 (1951).

(3) J. R. Marrack and E. S. Orlans, *Brit. J. Exptl. Pathol.*, **35**, 389 (1954).

(4) S. J. Singer, *Proc. Natl. Acad. Sci. U. S.*, **41**, 1041 (1955).

(5) T. Katsura, *Japan. J. Exptl. Med.*, **25**, 167 (1955).

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

(7) A. F. S. A. Habeeb, H. G. Cassidy, P. Stelos and S. J. Singer, *Biochim. Biophys. Acta*, **34**, 439 (1959).

(8) K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, 1945, pp. 141–143, 262–264.

(9) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947).

(10) F. W. Putnam, "The Proteins," Ed. H. Neurath and K. Bailey, Vol. I, Part B, Academic Press, Inc., New York, N. Y., 1953, pp. 893–972.

(11) A. Nisonoff and D. Pressman, THIS JOURNAL, **79**, 1616 (1957).

(12) D. Pressman and L. Pauling, *ibid.*, **71**, 2893 (1949).

(13) D. Pressman, A. L. Grossberg, L. H. Pence and L. Pauling, *ibid.*, **68**, 250 (1946).

plications inherent in precipitation of a large polyionic antigen by the large polyionic antibody.

We have now investigated the nature of the site of antibody produced in rabbits against the positively charged A_p hapten. We have reduced the number of negative charges on the antibody by esterification of carboxyl groups and have looked for effects in the site. The same reaction has also been applied to antibody against the negatively charged X_p hapten.

The reagent chosen was diazoacetamide (DAA), which reacts with protein carboxyls under mild conditions and has been reported to be quite specific; the only other group said to be attacked under the conditions employed is sulfhydryl.[14] Rabbit γ-globulin appears to have 2 sulfhydryl groups but these are masked, becoming available for reaction (with silver ion) only after denaturation with dodecyl sulfate.[15] Protein carboxyl is esterified by DAA and such treatment of anti-A_p antibody alters the antibody site itself so that it cannot bind hapten.

Experimental Methods

Preparation of Haptens.—Preparation of *p*-aminophenyltrimethylammonium chloride, *p*-(*p*-hydroxyphenylazo)-phenyltrimethylammonium chloride, *p*-hydroxyphenyl-A_p and I^{131} labeled *p*-iodobenzoic acid has been described previously.[13,16] The preparation of I^{131} labeled *p*-iodophenyltrimethylammonium chloride was similar to that of the *p*-iodo^{131} benzoic acid. The unlabeled compound was prepared by methylation of *p*-iodoaniline with excess methyl iodide in an ethanol–water mixture containing sodium carbonate. The slightly soluble iodide salt was converted to the chloride by treatment with silver oxide and then hydrochloric acid. The chloride was crystallized from ethanol.

Anal. Calcd. for $C_9H_{13}NICl$: Cl, 11.9. Found: Cl, 11.7, 11.8. M.p. 195–197° dec.

The exchange reaction was carried out at 150–160° for 18 hr. using 3 to 5 mg. of salt and 1 to 2 mc. I^{131}. The product was isolated as the iodide and converted to the chloride. The purification process was repeated until constant specific activity was obtained. Preparations had a specific activity of about 6 mc./m*M*.

Other haptens, or intermediates used in the above preparations, were commercial products of reagent grade.

Preparation of Diazoacetamide (DAA).—Glycine methyl ester was prepared according to the method of Curtius and Gobel.[17] Methyldiazoacetate was prepared in 85 to 95% yield from the glycine methyl ester according to the method of Womack and Nelson.[18] Diazoacetamide was prepared by ammonolysis in 10 *N* NH_4OH of the methyl diazoacetate for 48 to 72 hr. at 40°. The mixture was aerated, lyophilized and then extracted with acetone. The product crystallized from acetone on cooling. Yields averaged 5 to 8%.

Protein Antigens.—The immunizing antigen for anti-A_p serum was made by diazotizing two portions of *p*-aminophenyltrimethylammonium chloride weighing 75 mg. and 150 mg. and coupling separately to 5.00 g. portions of Bovine-γ-globulin (Pentex Co. Fraction II) in *p*H 9.5 borate buffer at 0° for 30 minutes. The azo proteins were brought to *p*H 8.5 and dialyzed against several changes of *p*H 8 buffered saline, combined and made up to 1% protein, with 0.2% phenol, for inoculation.

The immunizing antigen for anti-X_p serum has been previously described.[6,19]

Antisera.—Preparation and pooling of an antiserum and preparation of the γ-globulin fraction from serum by the method of Kekwick[20] have been described.[19] In all experiments the γ-globulin fraction of an antiserum pool or normal rabbit serum was utilized. "Anti-A_p" and "Anti-X_p" refer to the γ-globulin fractions containing these antibodies, respectively, and not to specifically purified antibodies.

Reaction of γ-Globulin with DAA.—γ-Globulin solution (6 to 8% protein) was dialyzed at 5° against 200 to 400 volumes of 0.15 *N* $NaClO_4$ buffered at *p*H 8 with 0.02 *M* sodium borate–boric acid buffer, to remove chloride ion which reacts with DAA. DAA was then added to a concentration of 0.35 to 0.45 *M* in DAA; a drop of *n*-caprylic alcohol reduced foaming. The reaction mixture was stirred continuously and 0.15 *N* $HClO_4$ was added slowly to reach and maintain *p*H 5.50. The acid flow was then adjusted to maintain the *p*H between 5.40 and 5.65. Heating from the magnetic stirrer motor produced a reaction temperature of 35 to 40°. No attempt was made to adjust the reaction temperature. After 1 to 1.5 hr. a duplicate amount of DAA was added and the reaction allowed to proceed, with subsequent addition of a third portion of DAA if necessary, until the desired degree of reaction was obtained after 2 to 4 hr. Esterification consumes hydrogen ion, allowing estimate of degree of reaction from the amount of acid added.[21] Following treatment, the mixture was brought to *p*H 7.5 and dialyzed at 5° for 18 to 40 hr. against *p*H 8–borate-buffered saline. Recovery of soluble protein varied from 65 to 90%, being less at the higher extents of reaction.

Equilibrium Dialysis.—The technique as described by Nisonoff and Pressman[19] was employed, except that all samples of a single run were dialyzed against the same large volume (30 to 60 ml.) of hapten solution, to assure that the free hapten concentration of the samples was the same.

Analytical Methods.—Concentration of protein was determined by optical density measurements at 280 mμ, utilizing as standard a rabbit γ-globulin solution of known nitrogen content. Concentration of the colored hapten, *p*-hydroxyphenyl-A_p, was determined by optical density measurements at 445 mμ, utilizing a standard curve and correcting for effect of γ-globulin solution on the optical density. Concentration of radioactive haptens was determined in a well-type scintillation counter, standardized daily with solutions of known concentration. A minimum of 10,000 counts was recorded for each sample, corresponding to a maximum probable counting error (corrected for background) of 1.2%. Concentration of standard solutions of labeled haptens was determined through use of standard curves of optical density *vs.* concentration—at 238 mμ for *p*-iodophenyltrimethylammonium chloride and at 248 mμ for *p*-iodobenzoate.

Titration of untreated and esterified γ-globulin was performed as described by Alexander and Cousens.[22] Carboxyl groups were estimated by the acid consumed in the range *p*H 2 to 5.8. Determinations were reproducible to ± 2% for a single protein preparation. An average value of 110 ± 5 titratable carboxyl groups per 160,000 gm. protein was found for several different samples of rabbit γ-globulin from normal serum or antiserum. The total number of free carboxyl groups in rabbit γ-globulin estimated from amino acid analyses has been reported[23] to be 121. DAA-treated samples always assayed as having carboxyl groups below 95 per 160,000 g. protein. Since the determination indicated variation of ± 5% in titratable carboxyl content of different samples of untreated γ-globulin, percentage esterification of a DAA-treated sample was calculated on the basis of the carboxyl content of its control untreated sample exposed to exactly the same procedures of dialysis and assay.

Results

Effect of DAA-Treatment of Anti-A_p on Hapten Binding.—The binding of haptens by anti-A_p was reduced following treatment of the antibody by DAA, as shown in Table I, in which the binding by

(14) P. E. Wilcox, Abstr. XIIth Internl. Cong. Pure and Appl. Chem., New York, N. Y., 1951, pp. 60–61; as quoted in ref. 10.

(15) G. Markus, personal communication.

(16) M. Blau, A. C. Johnson and D. Pressman, *Internat. J. Appl. Radiation and Isotopes*, **3**, 217 (1958).

(17) Curtius and Gobel, *J. prakt. Chem.*, **37**, 159 (1888).

(18) E. B. Womack and A. B. Nelson, "Organic Syntheses," Vol. III, John Wiley and Sons, Inc., 1955, pp. 392–393.

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

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

(21) M. S. Doscher and P. E. Wilcox, *Fed. Proc.*, **18**, 217 (1959).

(22) P. Alexander and S. F. Cousens, *Biochem. Pharmacol.*, **1**, 25 (1958).

(23) E. L. Smith, M. C. McFadden, A. Stockell and V. Buettner-Janusch, *J. Biol. Chem.*, **214**, 197 (1955).

TABLE I

EFFECT OF DAA TREATMENT ON THE BINDING CAPACITY OF ANTI-A_p ANTIBODY

γ-Globulin preparation	Hapten	Sample[a]	Carboxyl esterified (%)	Hapten conc. bound ($\times 10^6$ M)[b]
1	I^{131} *p*-iodophenyltrimethylammonium[c]	Untreated	0	33.9 ± 0.2
		DAA treated	14 ± 2	18.0 ± .2
		Normal γ-globulin	0	0.86 ± .03
2	*p*-Hydroxyphenyl-A_p[d]	Untreated	0	21.1 ± .5
		DAA treated	12 ± 2	11.2 ± .8
		Normal γ-globulin	0	2.9 ± .2

[a] Binding measurements performed at protein concentration of 20 mg./ml. [b] Average of duplicate determinations with average deviation; binding by antibody corrected for the binding by normal γ-globulin. [c] Free hapten concentration: 7.19×10^{-6} M. [d] Free hapten concentration: 4.04×10^{-6} M.

the treated globulins is compared with that of the untreated controls. Two different haptens, I^{131} labeled *p*-iodophenyltrimethylammonium ion and *p*-hydroxyphenyl-A_p, were used and the same effect was observed for each.

Protection of Anti-A_p by Hapten During Treatment with DAA.—Anti-A_p was treated with DAA in the presence of hapten to determine if the reduced hapten binding demonstrated by DAA-treated anti-A_p was due to an attack on the antibody site itself or on other parts of the molecule. A separate sample of anti-A_p was treated with DAA in a parallel manner with no hapten present. Assay revealed that samples esterified with and without hapten (antibody "protected" or "unprotected") were esterified to approximately the same extent (Table II). Following dialysis to remove excess hapten, binding measurements were made on the protected and unprotected samples and on untreated anti-A_p. The results (Table II) reveal that the presence of hapten during DAA treatment reduced the effect of this treatment on hapten binding. Thus, in a preparation esterified to 13%, *p*-hydroxyphenyl-A_p at 5×10^{-4} M prevented completely the 47% reduction in binding shown by the unprotected DAA treated anti-A_p. In the other anti-A_p preparations, in which esterification was more intense, with a correspondingly greater reduction in binding by the unprotected samples, protection by phenyltrimethylammonium ion and by I^{131} labeled *p*-iodophenyltrimethylammonium ion was readily apparent, although incomplete.

DAA Treatment of Anti-X_p.—DAA treatment did not affect the binding of I^{131} labeled *p*-iodobenzoate by anti-X_p (Experiment 1, Table III). To assure that conditions of DAA-treatment of anti-A_p and anti-X_p were comparable, mixtures of the two antibody-containing γ-globulin fractions were treated. Binding measurements on these treated mixtures, compared to those of the untreated mixtures (Table III, Experiments 2 and 3), show that the binding of hapten by DAA-treated anti-A_p was markedly reduced—to 37 and 33%, respectively, of the binding shown by the untreated mixture. In contrast, binding by DAA-treated anti-X_p in the mixtures was not reduced. The increase in hapten binding of about 15% by DAA-treated anti-X_p is also shown by DAA-treated normal rabbit γ-globulin (Table III, Experiment 4). Binding

TABLE II

PROTECTION OF ANTI-A_p ANTIBODY BY HAPTENS AGAINST ACTION OF DAA

Expt. no.	Hapten present	DAA treatment	Carboxyl esterified (%)	Concn. Ap hapten bound ($\times 10^6$ M)[a]
	None	None	0	21.1 ± 0.5
1[b]	None	Treated	12	11.2 ± .8
	p-Hydroxyphenyl-A_p-(5×10^{-4} M)	Treated	13	21.1 ± .1
	None	None	0	35.5 ± .05
2[c]	None	Treated	27	11.7 ± .05
	Trimethylphenylammonium chloride (1×10^{-3} M)	Treated	24	24.2 ± .05
	None	None	0	32.24 ± .05
3[c]	None	Treated	18	13.74 ± .02
	p-Iodophenyltrimethylammonium chloride (5×10^{-3} M)	Treated	18	26.03 ± .25

[a] Average of duplicate determinations with average deviation; values corrected for binding by normal γ-globulin. Protein concentration, 20 mg./ml. [b] *p*-Hydroxyphenyl-A_p used for binding measurements; free concentration: 4.04×10^{-6} M. [c] I^{131} labeled *p*-iodophenyltrimethylammonium chloride used for binding measurements; free concentration: experiment 2, 7.45×10^{-6} M; experiment 3, 5.75×10^{-6} M.

TABLE III

EFFECT OF DAA TREATMENT ON THE HAPTEN BINDING OF MIXTURES OF ANTI-A_p AND ANTI-X_p ANTIBODIES

Experiment	Sample[a]	Carboxyl esterified (%)	Concn. Ap hapten bound ($\times 10^6$ M)[b]	Concn. I^{131} *p*-iodobenzoate bound[b] ($\times 10^6$ M)
	Anti-X_p untreated	0		6.63
1[c]	Anti-X_p, DAA treated	17 ± 2		6.70
	Normal γ-globulin			0.19
	Anti-X_p Anti-A_p, untreated	0	35.5	5.00
2[d]	Anti-X_p Anti-A_p, DAA treated	27 ± 3	11.7	5.80
	Normal γ-globulin	0	2.30	0.64
	Anti-X_p Anti-A_p, untreated	0	26.4	6.20
3[e]	Anti-X_p Anti-A_p, DAA treated	24 ± 3	9.7	7.05
	Normal γ-globulin	0	3.70	0.35
	Normal γ-globulin, untreated	0	0.64[g]	0.37
4[f]	Normal γ-globulin, DAA treated	30 ± 2	0.40[g]	0.91

[a] Binding measurements performed at protein concentration of 20 mg./ml. [b] Average of duplicate determinations with average deviation of ± 3%; binding by antibody corrected for binding by normal γ-globulin. [c] Free hapten concentration: 3.71×10^{-6} M. [d] A_p hapten: I^{131} labeled *p*-iodophenyltrimethylammonium chloride; free concentration: 7.45×10^{-6} M; free I^{131} labeled *p*-iodobenzoate concentration: 3.21×10^{-6} M. [e] A_p hapten: *p*-hydroxyphenyl-A_p; free concentration, 4.30×10^{-6} M; free I^{131} labeled *p*-iodobenzoate concentration, 4.32×10^{-6} M. [f] A_p hapten as in experiment 2; free concentration, 5.75×10^{-6} M; free I^{131} labeled *p*-iodobenzoate concentration, 4.67×10^{-6} M. [g] Measured with another preparation.

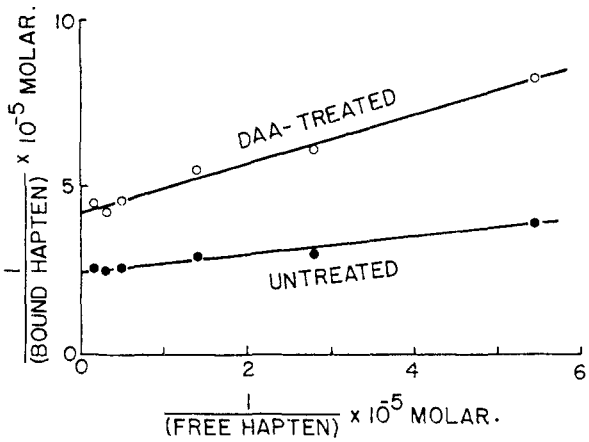

Fig. 1.—Binding of I^{131} labeled *p*-iodophenyltrimethylammonium ion by DAA-treated and untreated Anti-A_p antibody at 5°, with the antibody preparation of experiment 1, Table I.

of I^{131} labeled *p*-iodophenyltrimethylammonium ion by normal γ-globulin was not appreciably affected by DAA-treatment of the protein.

Combining Constants of DAA-treated and Untreated Anti-A_p.—The extent of the binding of I^{131} labeled *p*-iodophenyltrimethylammonium ion at several levels of free hapten concentration was determined with untreated anti-A_p and with anti-A_p esterified to 16%. The binding results are plotted in Fig. 1 as the reciprocal of the free hapten concentrations against the reciprocals of the bound hapten concentrations. As previously discussed[18] the ordinate intercept of such a plot provides an estimate of the reciprocal of the total number of binding sites present in the sample; the reciprocal of free hapten concentration at which half of these total number of sites are occupied gives an estimate of K_0' the average combining constant for the sites in the sample. From Fig. 2, it was calculated that the concentration of combining sites in the 20 mg./ml. protein solutions is 41.5×10^{-6} *M* for the untreated anti-A_p, and 24.1×10^{-6} *M* for the DAA treated anti-A_p, corresponding to destruction of 42% of the sites. The K_0' values are 8.7×10^5 and 5.6×10^5, respectively, showing that esterification reduced the binding strength of the remaining sites by 35%.

Dependence of Destruction of Sites on the Extent of Esterification of Anti-A_p.—Results of binding measurements on all anti-A_p samples treated with DAA are plotted in Fig. 2 as percentage reduction in binding (binding by the untreated samples taken as 100%) on a logarithmic scale, against per cent esterification of carboxyl. The destruction of binding sites progresses as more protein carboxyl is esterified.

Hydrolysis of DAA Treated Anti-A_p.—DAA-treated and untreated anti-A_p samples were hydrolyzed under two sets of conditions of *p*H and temperature to determine if hydrolysis of ester linkages would lead to recovery of binding capacity. The pertinent details and the data on binding measurements are included in Table IV. Hydrolysis of ester groups was accompanied by about 20% recovery of the binding capacity lost by the original

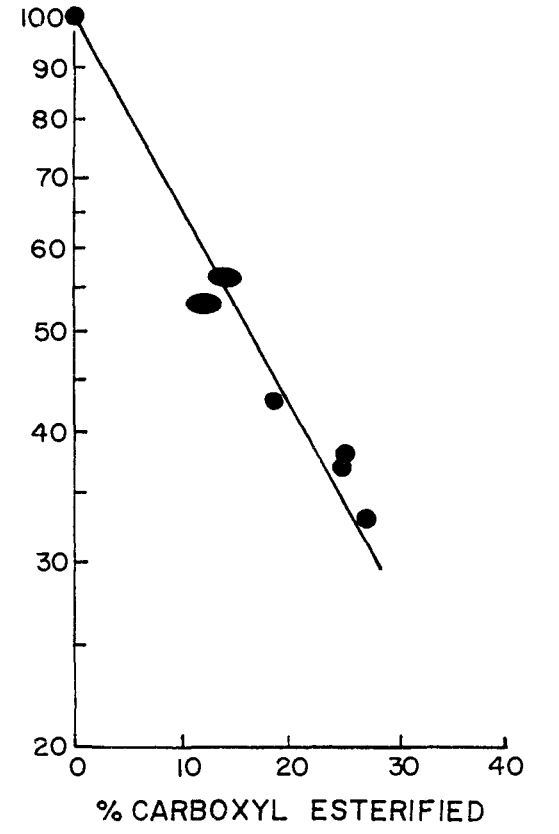

Fig. 2.—The effect of esterification of antibody protein carboxyl on the binding capacity of Anti-A_p antibody; diameter of a point represents the estimated reliability of the extent of esterification.

DAA treatment. The hydrolysis procedures did not cause loss of binding capacity in the untreated anti-A_p.

TABLE IV

EFFECT OF HYDROLYSIS OF DAA-TREATED AND UNTREATED ANTI-A_p ANTIBODY[a] ON HAPTEN BINDING CAPACITIES

DAA treatment	Hydrolysis[b]	Carboxyl content (groups/160,000 g.)	Conc. hapten bound ($M \times 10^6$)[c]
None	None	101	40.0[d]
Treated	None	85	21.3[d]
None	*p*H 11.0; 5°	105	41.7 ± 0.5
Treated		112	25.4 ± .6
None	*p*H 9.7; 25°	•	40.5 ± .2
Treated		115	24.6 ± .4

[a] The samples used were aliquots of the untreated and DAA treated Anti-A_p proteins of experiment 1, Table I. [b] 30 mg. of protein samples in 1.2 ml. were dialyzed for 18 hr. against 500 ml. of borate buffer under the conditions indicated. [c] 20 mg./ml. of protein concentration. Hapten: I^{131} labeled *p*-iodophenyltrimethylammonium chloride; free concentration, 14.5×10^{-6} *M*; values are averages of duplicate determinations, with average deviation indicated, corrected for binding by normal γ-globulin; binding was measured at *p*H 8.0. [d] Calculated from the curves of Fig. 2 for binding at the free hapten concentration used. • Not measured.

Evidence that DAA Alkylates Only Carboxyls.—Rabbit γ-globulin was esterified to 30% by DAA and tested to determine whether any alkylation of other reactive groups occurred. No decrease in the amino content was detectable by van Slyke

nitrogen determination,[24] indicating that less than an estimated 3 to 4% of the amino groups reacted. No shift in the tyrosine light absorption curve was observed, indicating that less than an estimated 5% of the tyrosines were alkylated.[22] No decrease in the degree of coupling of diazotized sulfanilic acid was found, suggesting that there was also little alkylation of imidazole by DAA. Titration of free sulfhydryl[25] indicated that one of the two masked sulfhydryls had been alkylated.

Discussion

Previous reports,[6,13] showed that anti-A_p antibody appears to have a negative charge in the site which accommodates the positive charge of the antigen and that anti-X_p antibody formed against a negative charge has a positive charge in the site. The present work emphasizes the difference in the antibody combining sites of the anti-A_p and anti-X_p antibodies. When a mixture of these antibodies is treated with DAA, with resultant esterification of carboxylate, only the anti-A_p antibody is affected, although the anti-X_p was esterified simultaneously in the same solution. The extent of esterification must have been identical for both antibodies. This is proof that a carboxylate or at least a moiety capable of reacting with DAA is present in the anti-A_p site, while no such grouping is present in the anti-benzoate site. That it is the attack on the site itself and not the non-specific alteration of the anti-A_p molecule which is responsible for the effect is further emphasized by the fact that esterification of anti-A_p antibody in the presence of the specific hapten results in a retention of antibody activity. Specific protection would not be obtained unless the attack were on the antibody site. That esterification is responsible for the loss of activity is indicated by the fact that binding activity can be partially recovered by exposing the altered antibody to *p*H 11, which hydrolyzes the ester groups.

These findings provide clear-cut evidence, implied by previous work,[11-13] that the specific sites in antibodies against different antigens have different amino acid composition. This does not differentiate between involvement of different parts of the same polypeptide or the associated tertiary structure or to variations in the sequence of amino acids in the chain at the region of the sites.

From the rate of decrease of binding activity with esterification—70% loss of activity when 30% of the carboxyls are esterified—it would appear that the carboxyl in the site is more easily esterified than some of the other carboxyls. Part of this loss of binding activity may be due to a non-specific effect as shown by the fact that when 16% of the carboxyls are esterified there is a 35% decrease in the value of K_0'. This non-specific effect may be partially electrostatic due to the increased positive charge of the antibody as a whole, resulting in decreased attraction or increased repulsion of the positive hapten. Steric effects due to an ester group close to the antibody site and distortion of the molecule as a whole may also contribute to the decrease in binding constant.

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

(25) Kindly performed by Dr. G. Markus by the method reported by G. Markus and F. Karush, THIS JOURNAL, **79**, 134 (1957).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN]

The *p*H Dependence of the Competitive Inhibition of Fumarase[1,2]

BY PAUL W. WIGLER[3] AND ROBERT A. ALBERTY

RECEIVED APRIL 1, 1960

In order to obtain further information on the structure and function of the active site of fumarase, the inhibitor dissociation constants with four competitive inhibitors, succinate, D-tartrate, L-tartrate and *meso*-tartrate, were determined over a range of *p*H values between *p*H 5.3 and 8.3. The dissociation constants of the inhibitor–enzyme compounds were determined using plots of $(S)/v$ *versus* (I) at each *p*H. From these data, the two acid dissociation constants (K_{aEI} and K_{bEI}) for each of the inhibitor–enzyme compounds and the dissociation constants of the inhibitor–enzyme compounds of the three ionized forms of the enzymatic site were calculated. It was found that the K_{aEI} and K_{bEI} values for the D and L-tartrate compounds of fumarase are lower than the corresponding values for the succinate compound, while the values for the *meso*-tartrate compound are higher. The K_{aEI} and K_{bEI} values are related to the fact that the hydroxyl groups of D and L-tartrate are in the *cis* configuration while the hydroxyl groups of *meso*-tartrate are in the *trans* configuration when the carboxylate groups are *trans*. The results are interpreted in terms of a model for the active site based on a *trans* dehydration of L-malate by fumarase.

Introduction

The divalent anions of several dicarboxylic acids are competitive inhibitors of pig heart fumarase.[4,5] In order to further elucidate the relationships between the spatial configuration of the inhibitor and the enzymatic site, experiments with the three isomers of tartrate and with succinate were carried out over a range of *p*H.

It has been demonstrated that there are two acidic groups which have a total effect on the kinetics of fumarase.[6] The *p*H dependence of the inhibitor dissociation constant (K_I) may be interpreted in terms of this model of the enzymatic site.[7] The

(1) This research was supported by grants from the National Science Foundation and from the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

(2) Presented in part before the 51st Annual Meeting of the American Society of Biological Chemists, Chicago, April 11–15, 1960.

(3) This investigation was carried out during the tenure of a National Institutes of Health Postdoctoral Research Fellowship from the National Heart Institute.

(4) V. Massey, *Biochem. J.*, **55**, 172 (1953).

(5) C. Frieden, Ph.D. Thesis, University of Wisconsin, 1955, p. 76.

(6) C. Frieden and R. A. Alberty, *J. Biol. Chem.*, **212**, 859 (1955).