SOME STUDIES OF THE CHEMICAL MODIFICATION OF ANTIBODIES

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SUMMARY

Chemical modification of the free amino groups of antibodies to six different antigens has been carried out. The antigens included three proteins, and one negatively charged, one positively charged, and one neutral, hapten. Acetylation of more than 40 % of the free amino groups of all antibodies resulted in a complete loss of capacity to precipitate their respective antigens, whereas guanidination of up to 75 % of the groups did not alter this capacity. Our conclusion is that the effect of acetylation is non-specific, independent of the nature of the antigen, and is probably due to a configurational change in the acetylated antibody molecules produced by intramolecular electrostatic repulsions. The dissociation of antigen–antibody bonds produced by alkaline pH may be attributable to a related mechanism.

INTRODUCTION

We have been interested in the possibility of detecting the presence of specific chemical groups in antigen (Ag) and antibody (Ab) reactive sites by modifying these molecules chemically and determining the accompanying effect on their activity[1–3]. For the purposes of a preliminary definition of the problem, we have investigated the modification of the free amino groups of Ab. Several different types of Ab were studied: three directed against the protein antigens bovine serum albumin (BSA), ovalbumin (OA), and ribonuclease (RNase); one directed against a negatively charged hapten, *p*-azobenzenearsonate ion (R); one directed against a neutral hapten, 2,4-dinitrophenyl (DNP); and one directed against a positively charged hapten, *p*-azotrimethylanilinium ion (TMA).

A fundamental difficulty which arises in all chemical modification studies with proteins is that if a loss of some biochemical activity is produced by a specific modification reaction, one must be able to determine whether this is indeed due to the blocking of specific groups within the reactive sites of the protein themselves, or whether it is the result of non-specific factors, such as a structural rearrangement of large segments of the protein molecule. In order to differentiate between these two effects, the following procedure proposed by SINGER[2] was employed, based on evidence which suggests that anti-R Ab sites contain a critical ϵ-amino group of lysine[4], and that anti-TMA Ab sites, on the contrary, probably contain a critical negatively charged group, very likely a carboxyl group[5]. The procedure, therefore,

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was to look for gross differences in the behavior of these two Ab upon chemical modification of their amino groups. If anti-R Ab were inactivated while anti-TMA Ab remained fully active upon blocking their amino groups, a specific inactivation at the reactive sites of the former Ab would be indicated. On the other hand, if both Ab were similarly inactivated, non-specific factors would appear to be involved.

Two specific modification reactions of amino groups were investigated: acetylation with acetic anhydride[6], and guanidination with O-methyl isourea[7]. Even partial acetylation resulted in a complete loss of capacity of all six Ab to precipitate their respective Ag, whereas extensive guanidination of all six Ab permitted them to retain essentially all of this capacity. We have concluded, therefore, that the inactivation produced by acetylation is of the non-specific type.

After these studies were completed, an interesting paper by NISONOFF AND PRESSMAN[8] appeared in which it was reported that the partial acetylation of Ab directed to the *p*-azobenzoate group resulted in a loss of capacity of the Ab to precipitate ovalbumin-azobenzoate but allowed essentially complete retention of the capacity to bind the small hapten, *p*-iodobenzoate. Their conclusion, in agreement with ours, is that the loss of Ab precipitability upon acetylation is a non-specific effect. Furthermore, however, they suggest that this non-specific effect is primarily due to the increased electrostatic repulsion between the acetylated Ab and the conjugated ovalbumin Ag molecules. Our results are inconsistent with this hypothesis, and we propose instead that upon acetylation, the Ab molecule undergoes a configurational rearrangement which interferes with the subsequent interaction with the large Ag molecule.

MATERIALS AND METHODS

Antigens

The proteins BSA, RNase, bovine γ-globulin (BG), and β-lactoglobulin (LG) were obtained commercially. OA was prepared from fresh hen eggs by the usual procedure[9]. For the study of the antihapten Ab, an injecting Ag was prepared by coupling the particular hapten to one protein, and a precipitating or test Ag was prepared by coupling the hapten to a second, serologically unrelated, protein. For anti-R Ab, an injecting Ag (RBG) was made by coupling diazotized *p*-arsanilic acid to BG[10]. The antisera so produced showed no significant titer against BG itself, and so RBG itself, as well as the trivalent dye, R_3' resorcinol[11], were used as test Ag. For anti-DNP Ab, the injecting Ag was DNP-BG, while the test Ag was DNP-BSA. The DNP-proteins were prepared with 2,4-dinitrofluorbenzene by the first procedure described by FRAENKEL-CONRAT, HARRIS AND LEVY[12]. For anti-TMA Ab, an injecting Ag was made by coupling diazotized *p*-amino (trimethylanilinium) chloride[5] to LG, while the test Ag was TMA coupled to BSA.

Antibodies

The hapten-conjugated proteins referred to above as injecting Ag, and also the proteins BSA, OA, and RNase, were administered to rabbits subcutaneously using the FREUND adjuvant technique. The γ-globulin fraction of the pooled highest titer antisera was precipitated with 1/3 saturated $(NH_4)_2SO_4$, and stored at 4° until needed, whereupon it was dissolved in and dialyzed against a nearly neutral buffer;

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this was phosphate buffer, pH 7.5, $\Gamma/2$ 0.1 in all cases except the anti-R system, for which maleate buffer, pH 7.0, $\Gamma/2$ 0.1, was used to avoid phosphate ion inhibition in this system[4].

Modification of Ab

Acetylation was carried out essentially as previously described[1,6]. Where low degrees of acetylation were required, it was found convenient to add small amounts of acetic anhydride by first preparing 10% solutions of it in dioxane. Guanidination[1,7] was performed at two pH values, 9.5 and 10.0 at 0° for 72 h, in order to vary the extent of conversion of amino groups. The number of amino groups remaining on the modified Ab was determined by ninhydrin analyses[3].

Ab activity determinations

For the purposes of this study, a relatively rapid assay was used. First, the amount of test Ag required to precipitate the maximum amount of specific unmodified Ab from the γ-globulin fraction was determined by Nessler N analyses. Second, this chemically modified γ-globulin fraction at the same total protein concentration was treated with the above-determined amount of Ag. The ratio of the amount of precipitate formed in the second case to the amount in the first was considered a measure of the fraction of precipitating capacity retained by the Ab after modification. No significant differences in precipitating capacity were found whether the precipitates were allowed to form for 40 or 72 h, which indicates that changes in precipitating capacity upon Ab modification can not be attributed to reduced rates of Ag–Ab reactions.

In view of the experiments of NISONOFF AND PRESSMAN[8], we specifically refrain from applying the term "inactivation" to a reduction in precipitating capacity of Ab.

RESULTS AND DISCUSSION

The reduction in precipitating capacity produced by the acetylation of Ab was very marked and quite similar in all six systems (Fig. 1). The acetylation of more than 40% of the amino groups of all six Ab resulted in a complete loss of precipitability, while even only 20% acetylation produced over a 60% loss of precipitability. On the other hand, guanidination of as many as 75% of the free amino groups of all six Ab produced no significant loss of activity (Table I).

Since the losses in precipitating capacity produced by acetylation of anti-R and of anti-TMA Ab are quite parallel, we infer that in these cases such losses are attributable to some auxiliary effects of acetylation rather than to the blocking of critical amino groups in the Ab sites. The similarity of the results obtained with all six Ab systems suggests that this conclusion applies generally. Up to this point, this conclusion is in accord with the interpretation of other workers[8,13,14], and is contrary to an earlier one from this laboratory[1,2].

What is the nature of these auxiliary effects of acetylation? One result of acetylation is the replacement, at neutral pH, of positively charged ammonium groups with uncharged acetamido groups, which increases the net negative charge of Ab. One conceivable explanation, therefore, is that the increased *intermolecular* electrical repulsion of acetylated Ab and Ag molecules is sufficient to overcome the specific

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binding forces of Ag and Ab. This interpretation was advanced by NISONOFF AND PRESSMAN[8]. There are several lines of evidence, however, which make this explanation untenable, the most directly pertinent of which is our observation that essentially

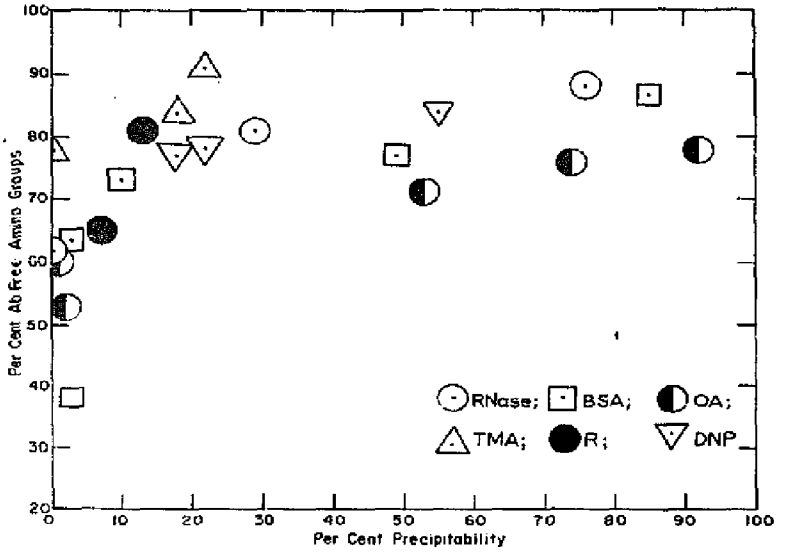

Fig. 1. The effect of acetylation of the NH_2 groups of different Ab molecules on their precipitability with homologous Ag, in different systems.

TABLE I

ACTIVITY OF GUANIDINATED ANTIBODIES

Ab	*Ab-free NH_2 groups* %	*Test Ag*	*Ppt. protein* mg	*Ab activity** %
Anti-RNase	100	0.04 mg RNase	0.68	100
	31		0.74	109
	24		0.75	110
Anti-BSA	100	0.10 mg BSA	0.44	100
	70		0.46	105
	28		0.55	124
Anti-OA	100	0.06 mg OA	0.64	100
	40		0.67	105
	25		0.72	112
Anti-TMA-LG	100	0.04 mg TMA-BSA	0.148	100
	30		0.142	96
Anti-DNP-BG	100	0.06 mg DNP-BSA	0.41	100
	67		0.33	80
	29		0.33	80
Anti-RBG	100	0.04 mg RBG	0.30	100
	44		0.35	116
	23		0.36	118

* In cases where the amount of precipitate is larger than with the untreated Ab, the diminished solubility of guanidinated Ab is probably the cause.

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the same loss of precipitability at pH 7.5 occurs with acetylated Ab to RNase as with acetylated Ab to OA. Since the isoelectric points of RNase and OA are 7.8 and 4.5, respectively, at pH 7.5 the former molecule carries a net positive, the latter a net negative, charge. Increasing the net negative charge of anti-RNase Ab should have increased its capacity to precipitate RNase, if intermolecular electrical interactions were of primary importance. These results suggest, rather, that the effect of acetylation is upon the Ab itself, independent of the nature of the Ag.

Our previous studies[3] have shown that acetylation and succinylation, as a result of changes wrought in the charge distribution on protein molecules and the *intramolecular* electrostatic repulsions so produced, can cause gross configurational changes in the protein molecules. We suggest that even partial acetylation causes such configurational changes in the Ab molecule in the neighborhood of the reactive sites, perhaps without affecting the sites *per se*[8], which changes are of such a nature as to hinder the binding of a hapten, or Ag site, when it is attached to a large protein molecule, but which may not hinder the binding of the small hapten itself. One might, for example, visualize the Ab sites as being partially invaginated by the acetylated, and thereby deformed, surrounding portions of the Ab molecule.

One may further suggest that the removal of the positive charge on the same amino groups of Ab by an increase of the pH of the solution, instead of by acetylation, might have the same effect on the Ab molecule and its capacity to precipitate with a large Ag molecule. The generally-observed dissociation of Ag–Ab bonds in alkaline solution might therefore be attributable to such a deformation of the Ab molecule, rather than to intermolecular repulsions of Ag and Ab molecules, or to the titration of specific critical groups within the Ab sites. If this speculation is valid, one might find, by analogy to the experiments of NISONOFF AND PRESSMAN, that an increase in alkaline pH might reduce the binding capacity of an Ab for its specific hapten when the latter was attached to a protein molecule, but have little or no effect on the Ab-binding capacity for the small hapten itself.

It is quite remarkable that extensive guanidination of the amino groups of Ab causes no appreciable inactivation. This demonstrates that the inhibition of precipitation produced by the acetylation of Ab is not due to the bulkiness of the acetamido groups *per se*, since the guanidino groups are virtually the same size. These results also afford no comfort for the view that amino groups are critically involved in Ab sites, particularly in anti-RAb sites[4], although they do not conclusively prove that they are not. It is possible, for example, that the critical amino groups might for various reasons be much less reactive towards O-methyl isourea than are the other amino groups of the Ab molecule. Such relatively unreactive groups have been encountered in other investigations[15].

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INCORPORATION OF ^{32}P INTO ISOLATED NUCLEI OF RABBIT APPENDIX: THE ROLE OF DEOXYRIBONUCLEIC ACID

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SUMMARY

1. Nuclei isolated from rabbit appendix could incorporate ^{32}P into DNA, RNA and organic acid-soluble phosphorus fraction (OASP) *in vitro*.

2. The removal of the bulk of DNA from the nucleus by pancreatic DNase abolished the incorporation into DNA and RNA almost completely, while that into OASP was reduced only to about 60%.

3. Appreciable restoration of the impaired incorporation activity was observed when salmon sperm DNA or yeast RNA was added to the nuclei treated with DNase. However, if an extensive removal of DNA had been achieved, the restoration occurred only in the case of RNA, and not DNA, of the nucleus on addition of exogenous nucleic acids.

4. Some indication was obtained that certain polyanionic compounds, such as chondroitin sulphate or hyaluronate, could replace the nucleic acids in restoring the impaired activity of DNase-treated nuclei, provided the action of DNase was not very extensive.

5. It was suggested that in the observed restoration added nucleic acids acted merely as polyanions which might eliminate the imbalance of electrostatic charges resulting from the removal of the bulk of DNA from the nucleus.

Abbreviations: ATP, adenosine triphosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; OASP, organic acid-soluble phosphate fraction.

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