AN ASPECT OF ANTIBODY HETEROGENEITY SUGGESTING CONFIGURATIONAL ISOMERISM [1]

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It is now widely assumed that the heterogeneity of antibody molecules directed against a single antigenic group and produced by a single animal is due to differences in their primary structure (Vansanten et al., 1964 ; Rockey et al., 1964 ; Doolittle et al., 1965 ; Lanckman, 1966). The present work describes a simple experiment which seems to show that , in addition to this heterogeneity, another one, resulting from the fact that antibody molecules of the same primary structure could present several different conformational states, may exist. This possibility has already been suggested for other globular proteins (Tanford, 1964 ; Markus, 1965).

Experimental

1) An immunological precipitate (formed at the point of equivalence or in the zone of antigen excess) consisting of tobacco mosaic virus (TMV) and non-fractionated anti-TMV antibodies produced

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by a single rabbit is divided into six batches. Each of these is incubated and gently stirred during 30 minutes in a 1 N acetate buffer at one of the following pH values : 4.5, 4, 3.5, 3, 2.5, 2 (the pH 2 buffer has a molarity slightly higher than 1) ; then a high speed centrifugation removes the non-dissociated precipitate and the released TMV. The supernatant is separated and the precipitate re-incubated in an identical volume of the same buffer for the same period of time. Each of these operations, repeated 4 - 5 times, promotes the release of a certain amount of antibody, which is measured with the Folin-Ciocalteu reagent after trichloracetic acid precipitation. The results are presented in figure 1. As can be seen, at each selected pH, the amount of antibodies extracted decreases very rapidly and reaches a value near zero after several extractions. At a given pH value a limited fraction of antibodies can be released ; the lower the pH the higher the total amount released (figure 1). An analogous result is

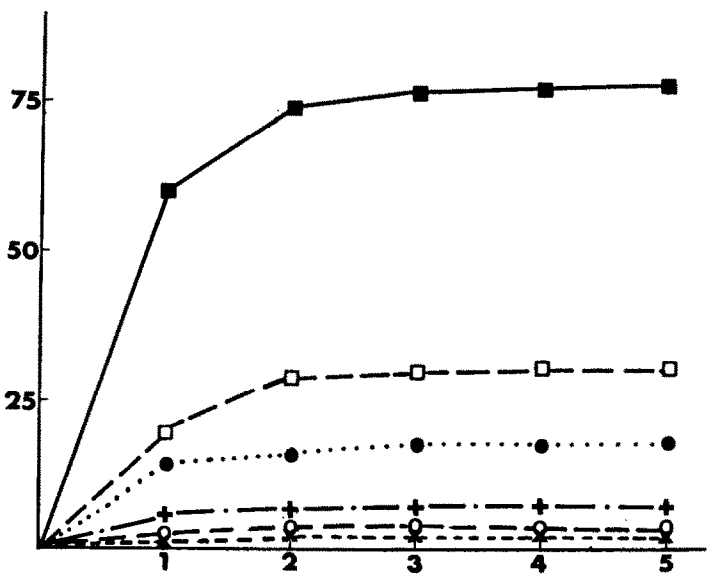

Fig. 1. Dissociation of anti-TMV antibodies at different pH.
Ordinate : Percentage released after the corresponding number of extractions (100 % = 2055 µg, initial amount).
Abscissa : Number of extractions (each extraction : 30 minutes, in 2 ml).
■-pH 2 ; □-pH 2.5 ; ● -pH 3 ; ✚ -pH 3.5 ; O-pH 4 ; X-pH 4.5.

obtained if the same batch of immune precipitate is extracted consecutively with buffers of increasing acidity : at any fixed pH value, the buffer promotes the release of only a fraction of the fixed antibody (this is achevied after several extractions by the method described above).

We can thus conclude that a decrease in the pH value strongly increases the dissociation constant of only a fraction of the antibodies fixed to the TMV.

2) This same experiment has been performed with rabbit antibodies of more restricted specificity : anti-TMV threonine + and anti-TMV threonine - antibodies[1]. The results are shown in figures 2^a and 2^b. No great differences were found between the behaviour of anti-TMV thr+ or of anti-TMV thr- and of total anti-TMV.

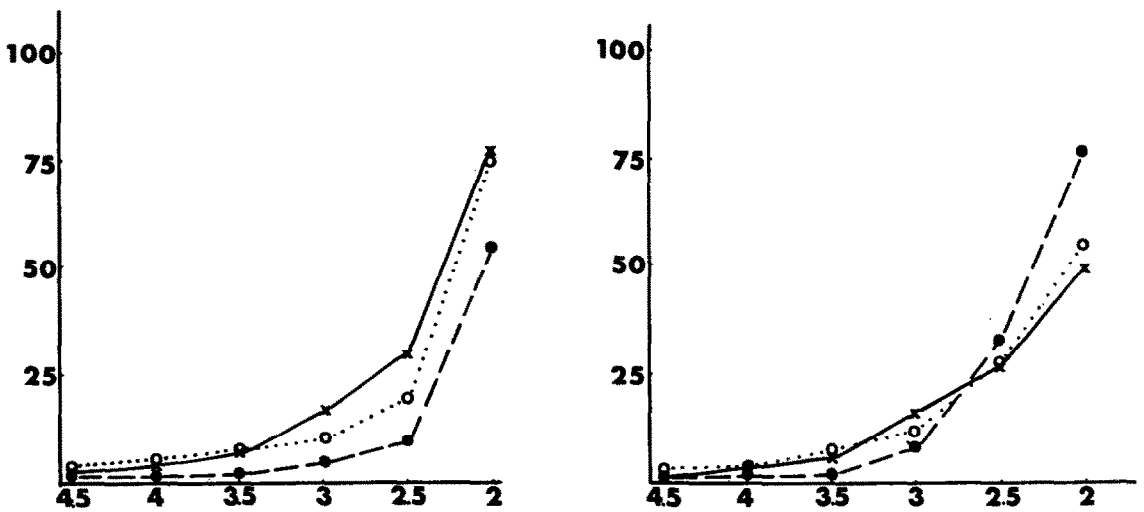

Fig. 2^a and 2^b. Dissociation of anti-TMV antibodies of restricted specificity (two experiments, with two rabbits).
Ordinate : Percentage released after 5 extractions of 30 minutes (100 % = initial amount).
Abscissa : pH of extractions.
x - anti-TMV antibodies ; ● - anti-TMV thr+ antibodies ; o -anti-TMV thr- antibodies.

1. Harris and Knight (1955) have shown that the presence or absence of the C-terminal threonine of TMV particles injected into rabbits determines the appearance of two different antibodies. These antibodies have been isolated in this laboratory (Vansanten et al., 1964). We shall call them anti-TMV threonine + (thr+) and anti-TMV threonine - (thr-).

Thus two antibodies of restricted and different specificities, amongst the anti-TMV antibodies, are not separated by this procedure ; each of these is itself divided into a number of fractions corresponding to the different pH values.

3) The antibodies dissociated between pH values 3.5 and 3 were dialysed back to neutrality and precipitated with TMV. Sixty % of these antibodies were precipitable with the antigen. The obtained complex was divided into batches which were then submitted to the action of the different buffers. As shown in figure 3, the dissociation diagram is very similar to the one obtained with unfractionated antibody ; (in this case, only one extraction of two hours was done with each batch).

Thus a separated fraction, when submitted to a new fractionation does not show properties characteristic of the pH at which it was originally isolated.

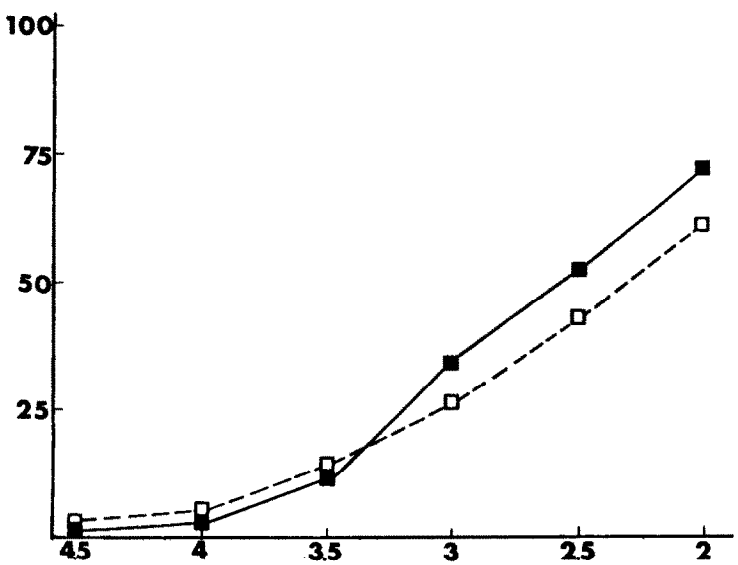

Fig. 3. Dissociation of the antibodies isolated between pH 3.5 and pH 3.
Ordinate : Percentage released (100 % = initial amount).
Abscissa : pH of extractions (at each pH, one extraction of 2 hours).
□ - total anti-TMV antibodies ; ■ - antibodies isolated between pH 3.5 and pH 3.

4) The Y-globulin of an immune serum rabbit was precipitated with 37 % saturated amonium sulfate. A part of this preparation was utilized to precipitate TMV. Another part was digested with papain according to Porter's method (Porter, 1959) and was passed through Sephadex G-200 in order to isolate the 3.5 S fragments. The complex between the so-obtained anti-TMV Fab fragments and TMV was submitted to a fractionation experiment according to the method mentioned above. This experiment was also performed with the precipitate consisting of TMV and non-digested antibody. The results, summarized in table 1, show that only a limited fraction of univalent fragments can be dissociated at each pH : the Fab fragments exhibit the same heterogeneous behaviour shown by the TMV antibodies.

Table 1. Dissociation of anti-TMV univalent fragments (Fab).

pH	First extraction	Second extract.	Third extract.	Fourth extract.	Fifth extract.	Total extracted µg	Total extracted %
		a) Non - digested antibody					
3.5	250	100	80	40	30	500	10
3	600	360	190	100	50	1300	26.5
2.5	1140	1060	300	150	80	2730	56
		b) Univalent fragments					
3.5	890	335	260	125	115	1725	35
3	1735	535	215	115	100	2700	55
2.5	2775	445	220	165	110	3715	76

In each case, initial amount is 4900 µg ; each extraction in 2 ml. ; extraction time: 30 min. ; amounts in µg.

The only differences between the fractionation of Fab fragments and of native antibody molecules are a slightly faster dissociation and the dissociation of larger amounts, at each pH value, observed for the fragments.

Discussion.

We are not able, on the basis of our experiments, to distinguish heterogeneity of antibody molecules from that of TMV elementary unit. The heterogeneity of the latter seems to be unlikely (Caspar, 1963).

The heterogeneity of antibody molecule cannot be the expression of differences in the primary structure of molecules, as one of the fractions separated during a first fractionation shows, during a second fractionation, the same range of heterogeneity as the total antibody[1]. This heterogeneity must concern the tertiary or quaternary structure of the molecules and may be explained by the hypothesis of configurational isomerism of antibody molecules. Such a hypothesis could be formulated as follows. Let us suppose that an amino acid sequence gives rise to various conformational states which are in dynamic equilibrium. Interaction with the TMV determinants could stabilize some of these states. Should these states show variable sensitivity to the ionic environment, dissociation by acid would show a heterogeneity of the type observed here. Brought back to pH 7, the antibody molecules which were previously stabilized in one configurational state by interactions with TMV would revert to the original equilibrium. A subpopulation isolated in a narrow pH interval and submitted again, after neutralization, to the same treatment as the total population would show the same heterogeneity as the original population. It has already been suggested that globular proteins may exhibit several different conformational states in dynamic equilibrium with each other (Tanford, 1964 ; Markus, 1965), and it has been shown by Schejter and George (1965) that a limited region of the cytochrome c protein can oscillate between two conformational states.

Recently Noelken et al. (1965) have suggested a model for the antibody molecule which would contain the fragments as separate entities joined to each other by a partially flexible structure. Such a situation would favor the occurence of different relative posi-

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1. It is important to note that the precipitates and the complexes were formed below the half-saturation point of TMV, so that complications arising from steric hindrance were minimized.

tions of the fragments of the antibody molecule fixed by its two sites on the TMV particle ; this would give rise to several different conformational states, which could have variable sensitivity to the ionic environment. The very similar bahaviour of Fab fragments and antibody molecules do not favor this hypothesis as being the cause of the binding heterogeneity. It seems more likely that the antibody molecules exhibit the supposed isomerism independently of such a phenomenon.

In summary, we would tentatively conclude that the antibody molecules present a heterogeneity due to conformational isomers, which is superimposed on the heterogeneity of amino acid sequences.

Acknowledgements

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