# THE EFFECT OF TETRANITROMETHANE ON THE ACTIVITY OF ANTI-TOBACCO MOSAIC VIRUS ANTIBODIES

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## 1. Introduction

The presence of two tyrosine residues in or near the combining site of several anti-hapten antibodies has been demonstrated by the affinity labelling experiments of Singer [1]. The tyrosine residues appear to be stable elements around which are organized the active sites, whose specificity presumably depends on the particular amino acid neighbours of these tyrosines.

Tetranitromethane (TNM), at low concentration and at nearly neutral pH, reacts specifically with tyrosine residues [2]. Its use should permit a rapid examination of the generality of tyrosines in relation to the combining site of antibodies of varying specificity or class and from different animals at various times during immunization. It is known that rabbit anti-dinitrophenyl antibodies, in which two tyrosines have been demonstrated in or near the combining site [1], can be partially inactivated by nitration with TNM under conditions where two nitrotyrosines per molecule of antibody are produced [3].

Experiments with antibodies from rabbits and mice immunized against tobacco mosaic virus (TMV) are reported in this note. In the two species studied, anti-TMV antibodies are inactivated by TNM. The conditions of this inactivation seem very different in early and late antibodies.

## 2. Materials and methods

### 2.1. *Preparation of antibodies and determination of their activity*

Rabbits (local stock) were immunized by four injections of 15 mg TMV, given at two day intervals in the vein of the ear or the foot pads. Mice (Balb/c) received four intraperitoneal injections of 100 $\mu$g TMV at two day intervals.

Antibodies were purified by two successive precipitations with TMV. After each precipitation, the antibodies were recovered by mechanical agitation in a solution of 0.15 M NaCl, which had been brought to pH 1.9 with HCl.

Antibody activity was determined by the addition of a large excess of TMV. Following centrifugation at 30,000 rpm, the amount of nonreactive antibody was measured by the absorbance of the supernatant at 280 nm. The appropriate correction was applied when part of the antibody tyrosines had been converted into nitrotyrosine [4].

Fab fragments were isolated according to the method of Porter [5]. Their activity was determined in the same way as whole antibodies.

### 2.2. *Action of tetranitromethane*

Nitration was carried out for 30 min at pH 8 following the procedure of Sokolowsky et al. [4]. As TNM is only slightly soluble, the concentrations actually attained during nitration were determined spectrophotometrically at 350 nm, following reaction with resorcinol.

Nitrotyrosine was determined spectrophotometrically at 428 nm and pH 8 [4].

Dilute solutions of nitrated antibody were concentrated on Amicon filters before being examined spectrophotometrically. Solutions were first subjected to a lengthy ultracentrifugation to remove antibody which had aggregated during nitration.



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### 3. Results and discussion

The clearest results were obtained by reacting TNM with solutions of anti-TMV antibody from rabbits and mice, isolated 9 and 12 days respectively after the start of immunization.

As can be seen in fig. 1, a large fraction of antibody molecules is inactivated in 30 min by a concentration of TNM in solution of 2 moles per mole of antibody; the complete inactivation of the antibody by a concentration of 20 moles of TNM per mole of antibody. At all concentrations of TNM used, inactivated antibody molecules (not bound by TMV after nitration) contain an average of two nitrotyrosines, i.e. one nitrotyrosine per active site, whereas antibody molecules which have remained active (bound by TMV) contain none.

When the nitration of antibody is carried out in the presence of an excess of TMV (whose accessible tyrosine had been nitrated previously), 75% of residues which would have been inactivated in the absence of the antigen remain active and free of nitrotyrosine.

These results suggested that there exists a tyrosine residue related to each combining site which is more accessible to TNM than any of the others and whose nitration inactivates the site. Confirmation of this hypothesis requires, in particular, verification that the same tyrosines are nitrated in all the inactivated molecules.

A second series of experiments was carried out on Fab fragments of antibodies from rabbits subjected to repeated injections of TMV for several months. In these experiments, the concentration of Fab was from 1 to 2 mg/ml and the quantities of TNM per ml were much greater than in the previous experiments. Most of the TNM was thus undissolved. Under these conditions, the proportion of Fab inactivated increased with the quantity of TNM added (fig. 2). In order to inactivate a large fraction of the Fab, two to three tyrosines had to be nitrated per fragment.

When the nitration was carried out in the presence of an excess of TMV, the inactivation was reduced by 65%, and the number of tyrosines nitrated was reduced by approximately one per Fab fragment.

Thus, with this material there does not appear to

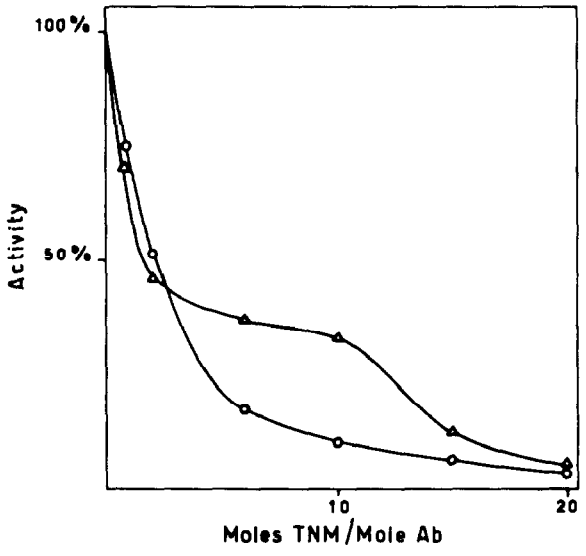


Fig. 1. The action of TNM on anti-TMV antibodies from rabbit (△) and mouse (○), obtained 9 and 12 days respectively after the beginning of immunization. Each point is the average of five experiments, in each of which 50 μg/ml of antibody were used; each antibody came from a single animal.

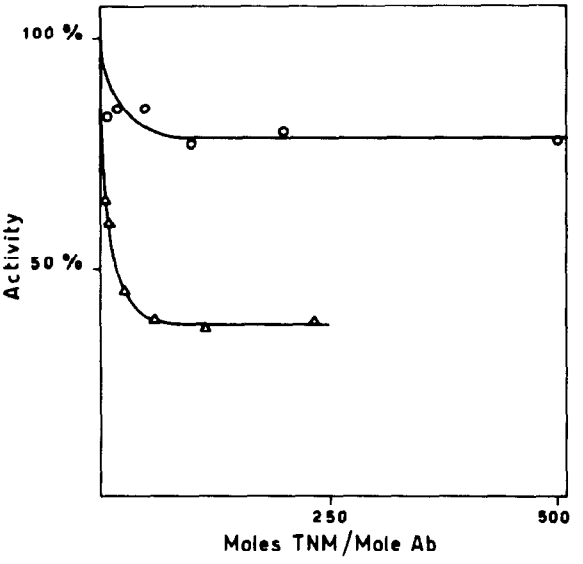


Fig. 2. Action of TNM on rabbit-anti TMV antibodies in the presence (○) and in the absence (△) of TMB. The antibodies were prepared from a mixture of serums obtained over a period of several months from a single animal.

be a particular tyrosine, essential for the function of the active site, and more accessible to TNM than other tyrosine residues. On the other hand, the protection of a Fab fragment against TNM inactivation is accompanied by the protection of one tyrosine residue from the effects of this reagent. As in the preceding experiment, the next step will be to localize the tyrosine which is protected by TMV and which appears to be essential for the functioning of the site. More recently, we have obtained similar results using both native antibodies synthesized 50 days after TMV injection and their monovalent fragments. Therefore the difference observed between the properties of early and late antibodies cannot be ascribed to papain cleavage and to the reformation of the resulting fragments.

Our tenative conclusion is that early antibodies contain in their active site a tyrosine which is more susceptible to nitration than antibodies synthesized after prolonged immunization.

## References

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