

## Chemical Modification of the Lysine-Amino Groups of Potato Virus X

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### SUMMARY

Potato virus X reacted with reagents commonly used for protein amino groups, and some of its properties were changed. 2,4,6-trinitrobenzenesulphonic acid, pyridoxal-5-phosphate and methyl picolinimidate altered its absorption spectrum; the last two altered its fluorescence spectrum, and the first two altered its electrophoretic mobility. These reagents did not necessarily inactivate the virus; preparations judged to contain two modified amino groups per protein subunit retained 50 to 100% of their initial infectivity. This supports the previous conclusion that PVX-Q, an infective product of PVX and an oxidized leaf phenol, contains modified lysine  $\epsilon$ -amino groups.

### INTRODUCTION

When potato virus X (PVX) is exposed to enzymically oxidizing chlorogenic acid, it is modified but not inactivated. No reactive -SH or terminal amino groups could be detected on the virus, and the modification was therefore interpreted as a reaction of *o*-chlorogenoquinone with a few of the 11 lysine  $\epsilon$ -amino groups in each virus protein subunit (Pierpoint, 1973). If this is correct, PVX should form infective derivatives with other reagents that react with protein amino groups. This possibility was therefore tested using pyridoxal-5-phosphate (PLP), 2,4,6-trinitrobenzenesulphonic acid (TNBS) and imidoesters, especially methyl picolinimidate (MEP).

These three reagents were chosen because they react fairly specifically with the amino groups of proteins, and the adducts they form are well known and can be estimated spectrophotometrically (Means & Feeney, 1971). However, the modified amino groups carry different charges at physiological pH: with MEP they are basic, with TNBS less so, and with PLP, as with chlorogenoquinone, they are acidic. It seemed likely, therefore, that with these reagents it might be possible to differentiate between the effects, on the infectivity of the virus, of structural and of ionic changes to the amino groups.

### METHODS

PVX was prepared from infected leaves of *Nicotiana tabacum* var. Xanthi-nc by sedimentation (Pierpoint, 1973), and its concentration estimated from its u.v. spectrum by assuming  $E_{1\text{ cm}}^{0.1\%}$ , uncorrected for light scatter, to be 3.5 (Bawden & Kleczkowski, 1959). The mol. wt. of its protein subunits was taken to be 30000 (Carpenter, 1972), and little if any of the lower mol. wt. form could be detected in samples examined electrophoretically in SDS gels by Dr Carpenter. Light scatter in virus suspensions was estimated by extrapolating the apparent extinction between 500 and 300 nm (Englander & Epstein, 1957).

PVX-Q was prepared as described previously (Pierpoint, 1973).

*Reaction of PVX with PLP.* PVX (13.4 mg), suspended in 1.0 ml of phosphate buffer (0.01 M, pH 7), was mixed with 0.5 ml of PLP (20  $\mu$ mol, pH 7), and incubated at 25 °C for 1 h. Two more lots of fresh PLP were added, each addition being followed by incubation. Two ml phosphate buffer (0.2 M, pH 7) was added and the mixture decolorized with a crystal of sodium borohydride. The solution was de-gassed in a Thunberg tube, diluted with 20 ml phosphate buffer (0.01 M, pH 7) and centrifuged for 2 h at 50000 g. The pelleted virus was resuspended in another 20 ml of dilute phosphate buffer and resedimented before being finally suspended in 3 ml of phosphate buffer (0.066 M, pH 7). A control preparation of PVX had the same treatment except that the PLP was incubated separately and added after being reduced with borohydride.

In some experiments, small amounts of PVX (< 1 mg) were treated with a large excess of PLP and dialysed against 0.05 M-phosphate buffer, pH 7. Dialysis had to be prolonged (> 50 h), and the buffer to be changed frequently to remove unreacted PLP completely.

*Reaction of PVX with TNBS.* PVX (12 to 15 mg) was incubated with 25 to 75  $\mu$ mol TNBS in 3 ml phosphate buffer (0.13 M, pH 7.5) at 20 °C. Additional TNBS (25 to 50  $\mu$ mol in 0.2 M-phosphate, pH 7.5) was added after 3 to 5 h and the incubation continued, sometimes overnight. The reaction was measured by the increase in  $E_{420}$  in a 2 mm spectrophotometer cell;  $\epsilon$ , the molar extinction coefficient for the lysine-TNBS-sulphite complex, was assumed to be  $1.31 \times 10^4$  (Goldfarb, 1966). Samples (0.3 ml) were taken at suitable times, diluted with 1.7 ml of phosphate buffer (0.2 M, pH 6.8) and dialysed against several changes of phosphate buffer (0.05 M, pH 7) for at least 24 h at 5 °C. Control samples of PVX, incubated without TNBS, were taken at the same time as the last experimental samples on each day.

The kinetics of the modification of PVX were measured by incubating 0.94 mg virus and 25  $\mu$ mol TNBS in 4 ml of phosphate buffer (0.16 M, pH 7.5) at 27 °C. The spectrum of the solution (300 to 450 nm) was scanned periodically using a solution without PVX as optical blank, and the spectrophotometer in its most sensitive position. The amount of unreacted amino groups at any time was measured as the difference between  $E_{345}$  and the maximum extinction that developed at this wavelength.

*Reaction of PVX with MEP.* PVX (8 mg) was incubated with MEP (96 to 100  $\mu$ mol) in 0.56 ml of phosphate buffer (0.05 M, pH 7) at 23 °C. After an appropriate time (0.1 to 4 h) more phosphate buffer (1.5 to 4 ml, 0.05 M, pH 7) was added and the samples dialysed against two changes of this buffer for at least two days at 5 °C. Sometimes the solutions were briefly centrifuged (700 g for 15 min) after dialysis. Control solutions of PVX were treated similarly except for the absence of MEP, and incubated for the maximum period in each experiment.

*Infectivity* was estimated from the number of local lesions produced on *Chenopodium amaranticolor* as described by Pierpoint (1973).

*Spectral measurements.* Absorption spectra were recorded on an Optica CF4 spectrophotometer, and fluorescence spectra on a Fluorispec SF1 spectrophotometer with the slits in the medium (8 nm band width) position and gain usually at 100.

*Electrophoresis* was done in the Tiselius cell of a Perkin-Elmer apparatus, model 38A, using 67 mm-phosphate buffer ( $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ , pH 7) as described by Pierpoint (1973).

*Buffers* were made as described by Pierpoint (1973). Methyl benzimidate HCl was made by the method of Hunter & Ludwig (1962) and MEP obtained from the Aldrich Chemical Company, Inc. Gossypol was kindly given by Dr W. A. Pons of the United States Department of Agriculture.

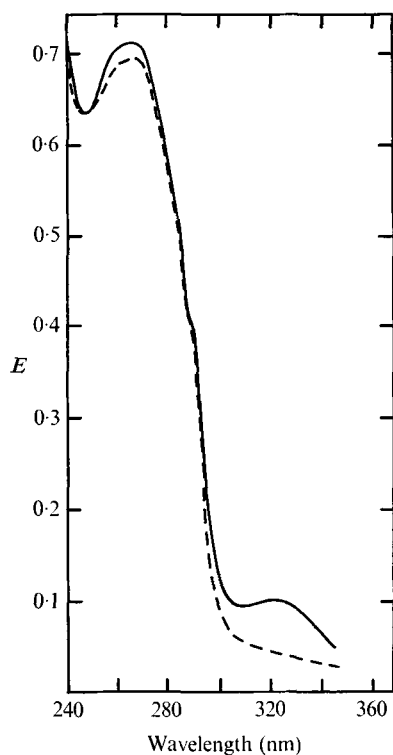


Fig. 1

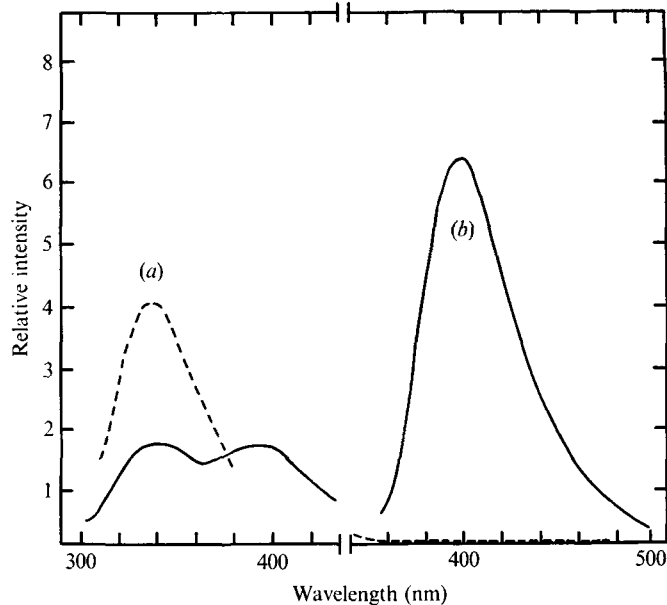


Fig. 2

Fig. 1. Extinction spectra of PVX (----) and PLP-modified PVX (—). The preparations were diluted in 7.5 vol. of phosphate buffer (0.05 M, pH 7) and their spectra measured in 2 mm cells.

Fig. 2. Fluorescence of PVX (----) and PLP-treated PVX (—). (a) Excited at 295 nm; solutions had  $E_{265}$  of 1.0. (b) Excited at 327 nm; solutions had  $E_{265}$  of 3.5.

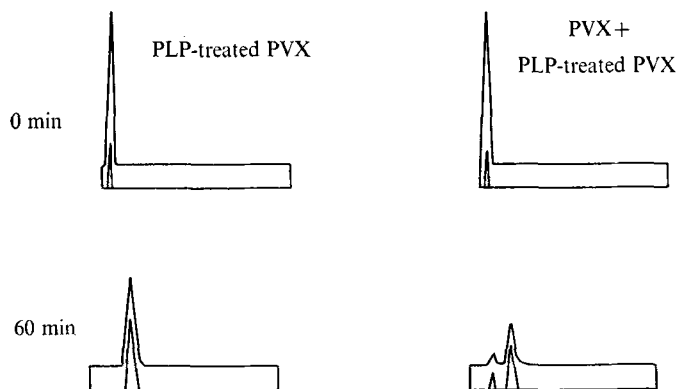
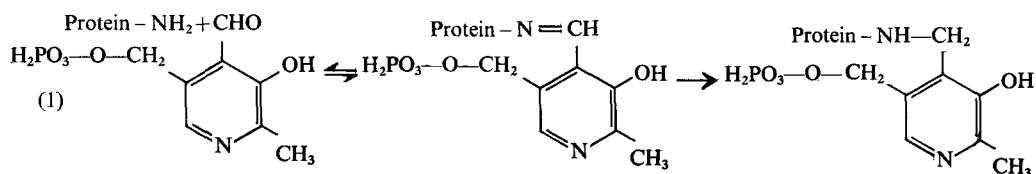


Fig. 3. Electrophoresis of PLP-treated PVX, and a mixture (6.5:1) of PLP-treated PVX and untreated PVX. The solutions had  $E_{265}$  of 6.1 and 6.4, respectively and were dialysed and analysed in the same sample of phosphate buffer (0.066 M, pH 7). Peaks produced by the ascending (left to right) boundaries were photographed after 0 and 60 min.

## RESULTS

*Modification of PVX by PLP*

The properties of PVX, re-isolated after treatment with PLP and reduction with sodium borohydride, indicate that it has been modified. Its u.v. spectrum has a peak at 325 nm (Fig. 1); its tryptophan-fluorescence observed at 340 nm, is decreased, and a longer wave fluorescence, maximally stimulated at 327 nm, introduced (Fig. 2); its electrophoretic mobility at pH 7 is approx. doubled (Fig. 3). These changes are characteristic of proteins whose amino groups have been modified by PLP (Means & Feeney, 1971; Cortijo & Shalteil, 1972; McKinley-McKee & Morris, 1972) according to equation (1); they do not occur if the PLP is reduced before it is added to PVX.

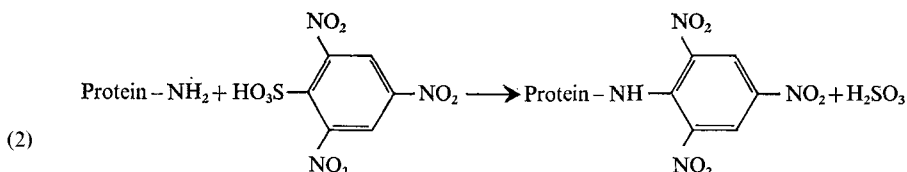


Assuming that  $\epsilon$  for lysine-bound PLP is  $10.7 \times 10^3$  at 325 nm (McKinley-McKee & Morris, 1972), the spectrum of modified PVX (Fig. 1) suggests that each protein subunit contains, on average, 0.85 molecules of PLP. Virus particles each contain more than 1000 subunits and, therefore, it is likely that very few particles are completely unmodified. Electrophoresis, which readily separates the two forms (Fig. 3), revealed no detectable unmodified virus in the PLP-treated preparation. However, this preparation was as infective as untreated PVX. In an assay in which untreated PVX at 0.33 and 0.066  $\mu\text{g/ml}$  produced 76 and 13 lesions/4 leaves of *Chenopodium amaranticolor*, the treated preparations produced 70 and 17 lesions.

Attempts to prepare PVX containing more than one PLP-modified lysine per subunit have so far been unsuccessful. They included incubating for longer periods, raising the pH to 8, and treating small amounts of virus with a 2300-fold excess of PLP.

*Reaction of PVX with TNBS*

When PVX was incubated with TNBS, an orange-yellow colour developed with extinction peaks at 345 and 425 nm. Dialysis decreased the 425 nm absorption peak (Fig. 4). These changes suggest a reaction between lysine  $\epsilon\text{-NH}_2$  groups and TNBS



and the formation of a product-sulphite complex which dissociates on dialysis (Goldfarb, 1966; Means & Feeney, 1971); the extent of the reaction can be estimated from the  $E_{345}$  assuming  $\epsilon$  for lysine-TBNS to be  $1.45 \times 10^4$  (Goldfarb, 1966). Electrophoresis also suggested the reaction of virus  $\text{-NH}_2$  groups; a preparation whose spectrum suggested that 7.1 lysine

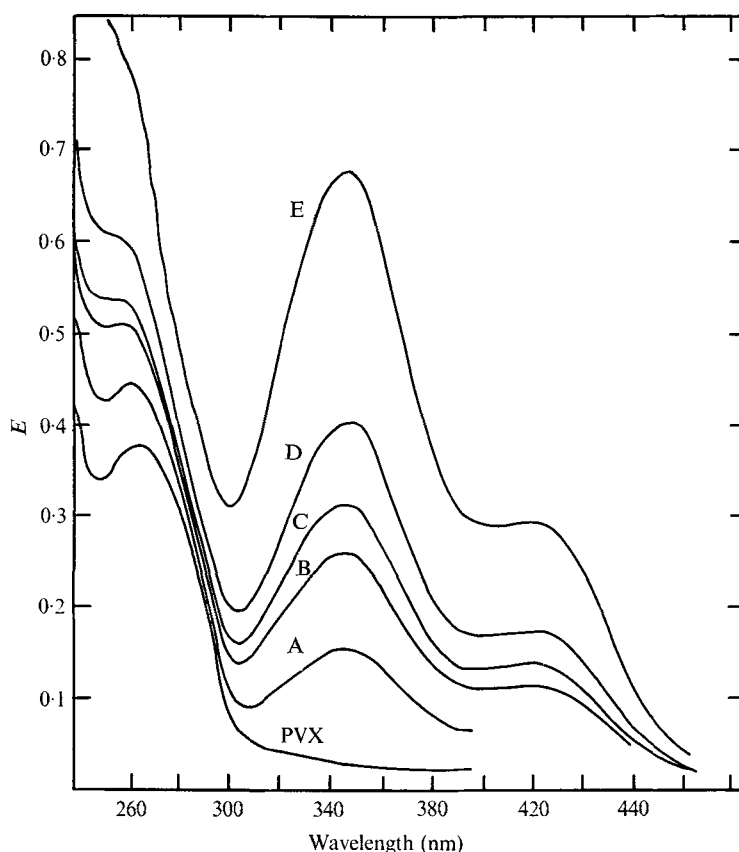


Fig. 4. Extinction spectra of PVX modified by TNBS. PVX (12 mg) was treated with TNBS (75  $\mu$ mol) essentially as described in Methods, and samples taken for dialysis at 75 (A), 162 (B), 220 (C) and 320 (D) min. Additional TNBS (50  $\mu$ mol) was added to the remaining solution before incubating it overnight (E). The reaction was estimated to modify 2.5 (A), 4.7 (B), 5.7 (C), 7.4 (D) and 12 (E) lysine residues per protein subunit.

residues per protein subunit had been modified, had a main component which migrated towards the anode 2.3 times as fast as untreated PVX.

Fig. 5. summarizes measurements of the infectivity of PVX preparations which had reacted with TNBS to different extents. Infectivity was not appreciably affected when two lysine residues in each protein subunit were substituted. However, it was progressively decreased as the number of reacted lysine groups increased, and was virtually abolished when five had reacted. The virus particles were also progressively disrupted as judged by their appearance in the electron microscope and their loss of streaming birefringence. The most extensively reacted preparations gave 9% more colour than expected from their lysine content, possibly due to a reaction of TNBS with the guanosine or terminal ribose (Azegami & Iwai, 1964) of exposed nucleic acid.

Attempts were made to follow the kinetics of the reaction between PVX and excess TNBS in conditions where the formation of the sulphite complex could be measured spectrophotometrically. The results (Fig. 6) show that 10% of the extinction develops comparatively quickly; the subsequent 40% more slowly and with approx. (pseudo) first-order kinetics, and the final 50% rapidly. This may indicate that each PVX subunit contains one amino

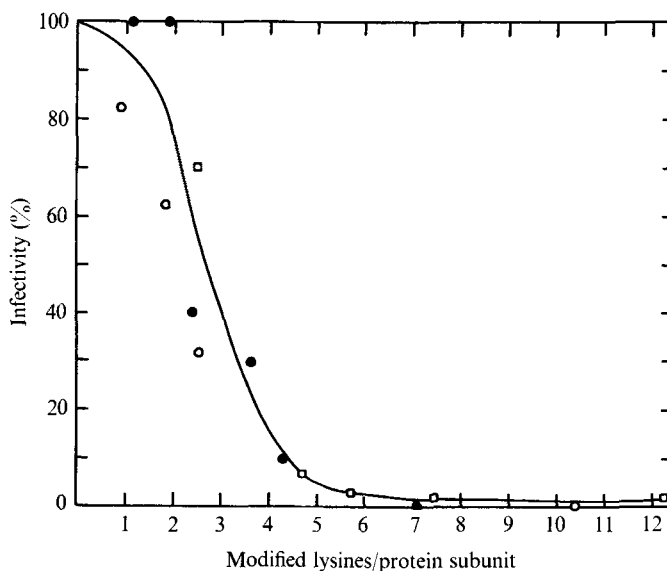


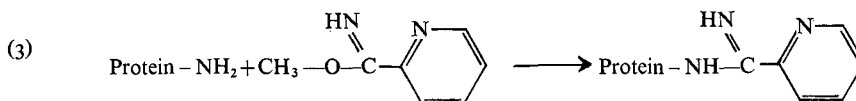
Fig. 5. Infectivity of TNBS-modified PVX. Lesion number in *Chenopodium amaranticolor* is expressed as a percentage of that induced by incubated but untreated PVX. Different symbols are used for results from four experiments.

group that is more reactive than the others, but that when half of the amino groups have reacted, the particle structure changes so as to accelerate the reaction of the rest. However this interpretation must be treated cautiously and qualitatively for a number of reasons. Thus the spectrum of the PVX-TNBS-sulphite complex differs from that of *N*-acetyl lysine-TNBS-sulphite (Goldfarb, 1966); the peak at 345 nm was usually 30% larger than expected and that at 420 nm 30% smaller, as if sulphite were partially dissociated from the complex. Moreover a small fine precipitate developed in the later stages of the reaction. This not only increased the apparent rate of reaction, but also increased the final apparent absorption and so affected the estimates of unreacted PVX.

#### *Reaction of PVX with imidoesters*

Methyl benzimidate (0.2 M suspension) probably reacted with PVX when incubated for 2 to 4 h in borate buffer (pH 8.3); the solution became more opalescent and the virus particles tended to aggregate. When re-isolated by dialysis and chromatography, the virus apparently absorbed more u.v. light, but this was partly due to increased scattering, and the spectrum had no marked peak by which modification could be measured. More than 80% of virus infectivity was lost.

Methyl picolinimidate (0.2 M, pH 7) also slowly precipitated PVX after 2 h at 23 °C. In shorter incubations it only slightly increased opalescence, but increased and sharpened the absorption spectrum of re-isolated PVX (Fig. 7). The spectral difference between the treated and untreated virus corresponds to that of MEP bound to lysine according to equation (3) (Benisek & Richards, 1968; Means & Feeney, 1971).



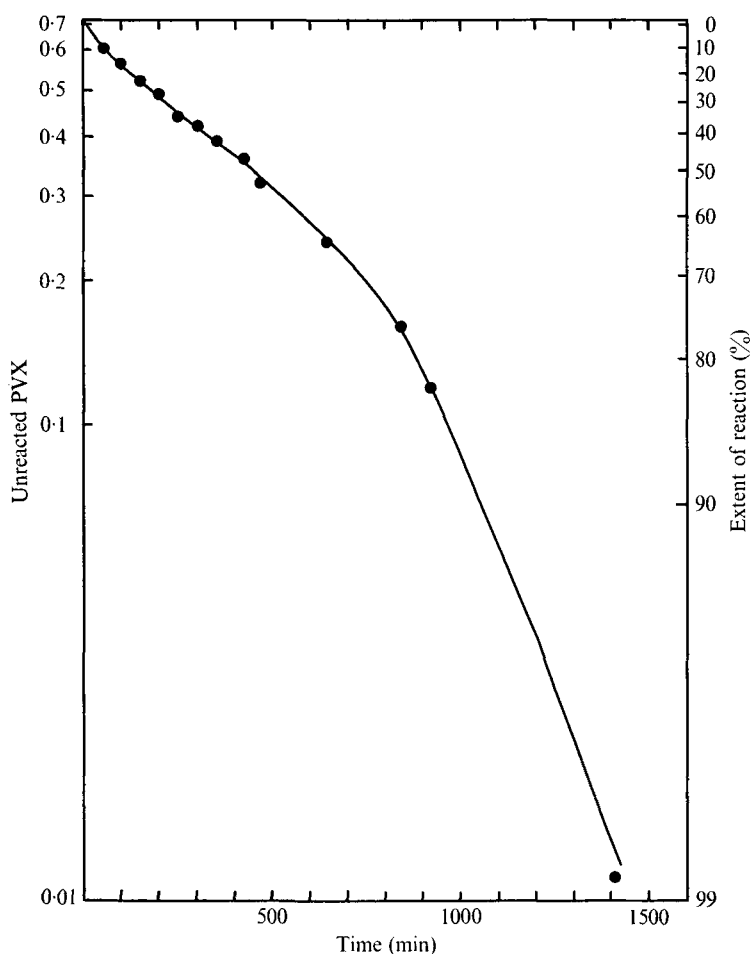


Fig. 6. Rate of reaction of TNBS with PVX. The reaction was followed spectrophotometrically at 345 nm in the conditions described in Methods.

The increased  $E_{262}$ , after correction for light scattering, can be used to make a rough estimate of bound MEP assuming  $\epsilon = 5700$  (Perham & Richards, 1968). From the  $E_{262}$  it is also possible to get a rough estimate of bound MEP when the treatment had precipitated some virus; the remaining virus can be estimated from the 'tyrosine-shoulder' at 290 nm, and the  $E_{232}$  due to virus estimated from the 262/290 ratio of PVX.

Experiments summarized in Fig. 8 show that the infectivity of PVX progressively diminished as successive lysine residues reacted; but, although it was halved by two substitutions, it was not completely abolished by more extensive reaction. More extensive reaction did not appreciably alter the appearance of the particles in the electron microscope, nor their electrophoretic mobility at pH 7, but it increased the tendency of the virus to aggregate, and it progressively quenched the tryptophan-fluorescence at 340 nm.

#### *Reaction of PVX with other reagents*

Dansylated derivatives of PVX were made by incubating virus with an excess (50- to 450-fold) of dansyl chloride (5-dimethylaminonaphthalenesulphonyl chloride) in alkaline

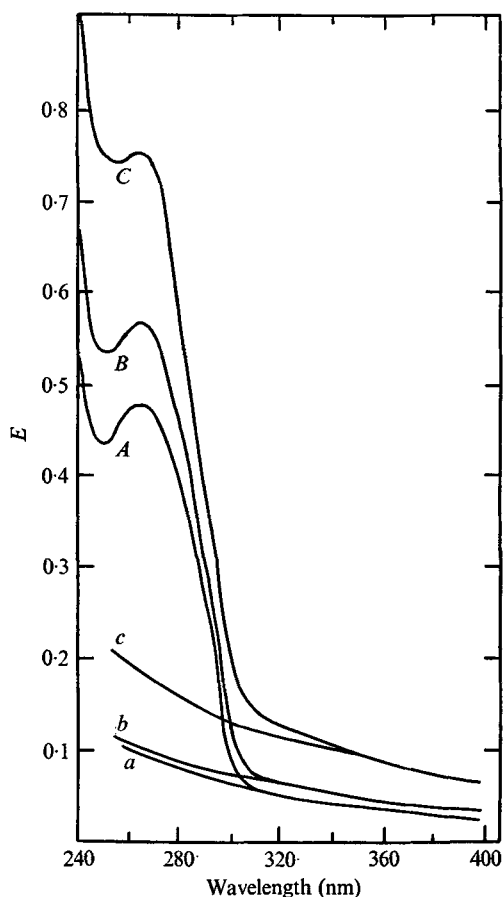


Fig. 7. Extinction spectra of PVX modified by MEP. PVX was treated for 0, 30 and 105 min, and the products, diluted in 13.5 vol. of phosphate (0.05 M, pH 7), were estimated to contain 0 (*A*), 2.7 (*B*) and 5.3 (*C*) molecules of bound MEP per protein subunit. The light scattered by these preparations (*a*, *b*, and *c*, respectively) was estimated before dilution.

solution (pH 8.3 to 9) of aqueous acetone (10%). Such preparations had a small peak at  $E_{330}$ , fluoresced at 520 nm, and contained a major component which moved about 40% faster than PVX on electrophoresis. Some preparations were slightly infective, although one, judged to contain 2.6 dansyl groups per protein subunit, was not. The relation between infectivity and the degree of dansylation could not be judged because alkaline acetone alone destroys 50 to 80% of virus infectivity, and the relatively small extinction coefficient of protein-bound dansyl groups ( $3.4 \times 10^3$ ; Means & Feeney, 1971) made their estimation unreliable.

Attempts to react the cottonseed aldehyde gossypol with the amino groups of PVX, in conditions in which it reacts with those of pepsinogen (Finlay, Dharmgongartama & Perlmann, 1973), have so far been unsuccessful.

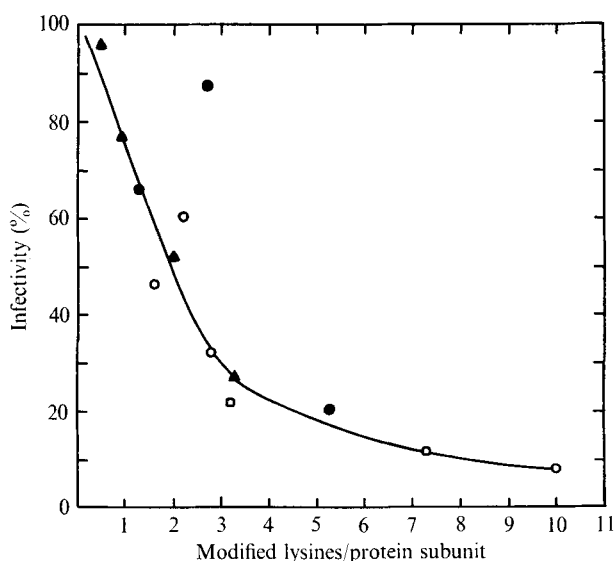


Fig. 8. Infectivity of MEP-modified PVX. Lesion number in *Chenopodium amaranticolor* is expressed as a percentage of that induced by incubated but untreated PVX. Different symbols are used for results from different experiments.

#### DISCUSSION

The present work shows that PLP, TNBS, MEP and dansyl chloride react with intact PVX. The specificity of these reagents (Means & Feeney, 1971), and the properties of the products suggest that combination is through the  $\epsilon$ -amino groups of exposed lysine residues in the virus. The work also shows that reaction of these residues does not necessarily remove infectivity. When only 1 or 2 residues per protein subunit are judged to have been modified, the bulk of infectivity (50 to 100 %) is retained. This is true whether the modification retains (PVX-MEP), or alters (PVX-TNBS, PVX-PLP) the basic charge of lysine; indeed, within the limits of the infectivity tests, TNBS-modified PVX was usually more infective than virus modified with MEP. Alterations to the basic charge are, however, likely to be more important as the virus is more extensively modified, and they may well explain the complete inactivation and particle disintegration that occurs when TNBS has reacted with half the virus lysine.

These conclusions are therefore consistent with, and add credibility to, the previous suggestion that infective PVX-Q contains quinone-modified lysine residues. They fix an upper limit of 2 to the number of such modifications per protein subunit. Attempts to measure the number of modifications directly by amino acid analysis have, so far, been discouraged by the partial lability of quinone-amino acid links during acid hydrolysis (Cranwell & Haworth, 1971).

It seems unlikely, *a priori*, that all the 11 lysine amino groups in each protein subunit of PVX are equally accessible and reactive. There is support for believing that one is more reactive than the others from the kinetics of the reaction with TNBS (Fig. 6), and also from the single substitution that occurs with PLP. One lysine residue in this strain of PVX (Carpenter, 1972), as in other strains (Koenig *et al.* 1970; Shepard & Secor, 1972; Tremaine & Agrawal, 1972; Tung & Knight, 1972), is exposed enough to be susceptible to hydrolysis by trypsin, when a large peptide of mol. wt. about 5000 is released, leaving an intact and partially infective particle. It is tempting to assume that it is this lysine group which is most

readily attacked by TNBS and PLP, and it is this one which primarily reacts with *o*-chloro-quinone to form PVX-Q. This assumption is currently being tested.

I am grateful to Mr G. Giancovich for his assistance.

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