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An Infective Pyridoxyl-derivative of Potato Virus X: PVX-PLP

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SUMMARY

The reduced initial product (PVX-PLP) of the reaction of pyridoxyl 5′-phosphate (PLP) and particles of potato virus X (PVX) contains about 1 (0·6 to 1·2) molecules of PLP per protein subunit and is infective. Hydrolysates of the protein contain N-ϵ-pyridoxyl-lysine. PVX-PLP reacts with chlorogenoquinone to 1/4 of the extent of PVX; similarly, PVX which has reacted with chlorogenoquinone (PVX-Q_1) binds only 1/3 to 1/5 as much PLP as does PVX. PVX-PLP contains two types of fluorescent subunit which can be separated by electrophoresis in SDS-acrylamide gels: one of these is fluorescent and is not degraded by brief exposure to trypsin, whereas the other is degraded to a smaller form which is also fluorescent. Tryptic digests of the protein from PVX-PLP contain at least two fluorescent peptides. It is argued that PLP reacts with two lysine ϵ-amino groups of PVX, one of which also reacts with chlorogenoquinone, and the other of which is recognized by trypsin.

Protein isolated from PVX reacts with up to 6 molecules of PLP. The conformation of the subunits in the intact virus apparently makes many ϵ-amino groups inaccessible to PLP.

INTRODUCTION

Potato virus X (PVX) has flexuous thread-like particles, 515 nm long, containing 6 % RNA enclosed in about 1400 helically arranged protein subunits (Bercks, 1970). The subunits are probably identical and, in the strain commonly used in our laboratory, have a mol. wt. of about 27000 and contain 11 to 12 lysine residues (our unpublished information). We have now investigated the reactivity of the ϵ-amino groups because of the evidence that they are involved in the reaction of PVX with o-chlorogenoquinone, the o-quinone produced during the enzymic oxidation of the leaf phenol, chlorogenic acid (Pierpoint, 1973; Pierpoint, Ireland & Carpenter, 1977).

Evidence, mainly spectrophotometric, was previously produced (Pierpoint, 1974) that the virus reacts with pyridoxal 5′-phosphate (PLP), picryl sulphonic acid (TNBS) and methyl picolinimidate (MP), three reagents known to have some specificity for amino groups (Means & Feeney, 1971). Reaction with TNBS was extensive and, when more than 2 amino groups had reacted, led to the loss of infectivity and virus structure. Reaction with MP was similar, but infectivity was not so completely destroyed. The reaction with PLP was, however, much more restricted; approximately one molecule of PLP reacted per subunit of protein, and the modified virus (PVX-PLP) retained its infectivity. This reaction resembles the reaction of PVX at pH 7 with chlorogenoquinone to give infective

PVX-Q_1 (Pierpoint *et al.* 1977). This resemblance has encouraged us to examine PVX-PLP further, to confirm that it contains a modified ϵ-amino group, and determine if this amino group is the one that is primarily modified by chlorogenoquinone, or the amino group that is readily recognized by trypsin. The reaction of the isolated PVX-protein with PLP was also studied to determine how far the limited reaction of the amino groups of the intact virus is a consequence of the configuration of the protein in virus particles.

METHODS

PVX. The strain of virus, its culture, isolation, estimation and infectivity measurements have been previously described (Pierpoint, 1973, 1974). Protein was isolated from virus suspensions, as well as from PVX-PLP by the acetic acid method as described previously (Carpenter, 1970).

Preparation of PVX-Q_1 and reaction of chlorogenoquinone with PVX-PLP. PVX and PVX-PLP were reacted with enzymically generated chlorogenoquinone essentially as described by Pierpoint *et al.* (1977).

Reaction of PLP with PVX particles. Conditions were similar to those used previously (Pierpoint, 1974). Typically, PVX (10 mg; 0·37 μmol of protein subunits) was suspended in 1 to 2·5 ml phosphate buffer (0·05 M; pH 7·5), and PLP (75 to 230 μmol dissolved in 1 to 3 ml of buffer and adjusted to pH 7·5) was added in 2 or 3 successive lots, and the mixture incubated at 25 °C for 15 to 30 min after each addition. The solution was cooled and decolorized with the minimum amount of solid sodium borohydride and the pH readjusted to 7 with dilute HCl. It was re-treated with a similar amount of borohydride and re-neutralized. A brief exposure to acid conditions (pH 6 with HCl) was occasionally used to decompose excess borohydride. The solution was then either de-gassed or dialysed overnight before the virus was separated from low molecular weight material either by permeation chromatography or centrifugation. Chromatography was done in phosphate buffer, 0·05 M, pH 7, either on a column of Sephadex G-50 (1·1 × 58 cm) or glass granules (1·2 × 107 cm; CPG-10, pore diam. 1400 Å) which had been treated with carbowax 20 M (Pierpoint *et al.* 1977). The reaction mixture was diluted 20-fold with phosphate buffer and then centrifuged at 50000 to 75000 *g* for 2 to 3 h; the pellet was resuspended in fresh buffer, re-diluted and re-sedimented.

Reaction of PLP with PVX-protein. Freeze-dried PVX-protein (10 mg) was reacted with a 160-fold molar excess of PLP in approx. 2 ml of phosphate buffer (0·05 M, pH 7·5) by the same procedure as were PVX particles. When protein was used without prior concentration by freeze-drying, the volume of the reaction mixture was twice as large.

Reaction of PLP with PVX-Q_1. (1) After formation, PVX-Q_1 was separated from reaction mixtures by chromatography on the column of glass granules and divided into two portions. One portion (7 mg; 0·26 μmol) was reacted with 60 μmol of PLP at pH 7·5, and reduced with ^{3}H-sodium borohydride; the second was reduced in the absence of PLP. Both samples of virus were de-gassed, diluted to 40 ml with phosphate buffer (0·05 M, pH 7), sedimented by centrifugation, and the pellets washed, re-suspended and re-sedimented. The washed pellets were re-suspended in 4 ml phosphate buffer, and samples taken for measurement of radioactivity. A control sample of PVX was pyridoxylated, reduced and washed under the same conditions, and its radioactivity measured.

(2) PVX-Q_1 (5 to 7 mg) was prepared and dialysed overnight to remove unoxidized chlorogenic acid. It was reacted, in the presence of oxidized, polymerized chlorogenic acid, with a 200 molar excess of PLP. It was reduced three times with sodium borohydride

to try to ensure complete reduction of the Schiff base formed between PVX and PLP, in the presence of reducible and autoxidisable polymerized-chlorogenic acid. The doubly reacted virus was dialysed overnight, separated chromatographically on CPG-10, and its PLP content estimated by fluorescence.

During the reaction of PVX with chlorogenoquinone, two control solutions were incubated, one containing PVX alone and the other chlorogenic acid plus polyphenoloxidase. After dialysis they were mixed, and reacted with PLP in the same conditions as PVX-Q_1. The virus, after chromatography, served as a control to estimate the PLP bound to PVX in the presence of oxidized but unbound chlorogenic acid.

Estimation of PLP bound to virus and virus-protein. Previously (Pierpoint, 1974) the PLP content of PVX-PLP was measured from its u.v. spectrum. Virus concentration was estimated from E_{265} by assuming $E_{1\,cm}^{0.1\%}$ to be 3·5; bound-PLP was estimated from E_{325} after subtracting the absorbance of a control sample of unmodified PVX at this wavelength, by assuming the molar extinction coefficient for N-ϵ-PLP-lysine to be 10700 at pH 7 (McKinley-McKee & Morris, 1972). As bound PLP contributes to the absorption at 265 nm as well as 325 nm, an attempt was made to refine this method. The ratio E_{265}/E_{325} for bound PLP was assumed to be 0·33, the same as the value measured on a synthetic preparation of N-ϵ-pyridoxyl-lysine. E_{265}/E_{325} was measured for each preparation of PVX, or, less satisfactory, assumed to have the average value of 12·5. Simultaneous equations were then constructed for the absorption of PVX-PLP at 265 and 325 nm, and their solution gave the approximate number of molecules of PLP bound per protein subunit.

The number of molecules of PLP bound per molecule of PVX-protein was similarly estimated from the u.v. spectrum and simultaneous equations expressing E at 290 and 325 nm in terms of the absorbance of PVX-protein and bound PLP. $E_{1\,cm}^{0.1\%}$ for the protein was taken as 0·89, and E_{290}/E_{325} as 22·2. The extinction coefficient for bound PLP at 325 nm was taken as 10700 and E_{290}/E_{325} assumed to be 0·32, the same as that measured on a preparation of N-ϵ-pyridoxyl-lysine.

The PLP content of 5 samples of PVX-PLP-protein measured by this method, was, on average, 80 % of the estimated PLP content of the intact PVX-PLP from which they were derived.

The relative amounts of bound PLP in different virus derivatives, and peptides eluted from electrophoretograms, was estimated from their relative fluorescence at 400 nm.

Estimation of chlorogenoquinone bound to virus. Chlorogenoquinone bound to PVX-PLP was estimated from the amount of quinic acid that could be liberated by saponification (Pierpoint *et al.* 1977).

Brief trypsin-treatments of virus particles and proteins. PVX, PVX-PLP, PVX-protein were tested for sensitivity to brief trypsin digestion (ratio by weight to protein 1:100) at 20 °C as previously described (Pierpoint *et al.* 1977). Reaction mixtures were examined in SDS-acrylamide gels which were prepared, run, stained and quantitatively scanned as described previously (Carpenter, Kassanis & White, 1977; Pierpoint *et al.* 1977).

Production and separation of 'tryptic-peptides'. PVX-protein, PVX-PLP protein and pyridoxylated PVX-protein were dissolved to give a 1 % (w/v) solution in ammoniumbi-carbonate solution (1 % w/v; pH 8·5) containing 0·01 % (w/v) diphenyl carbamoyl chloride-treated trypsin, and incubated for 3 h at 37 °C. In some experiments four times as much trypsin was added in portions, and incubation continued for 24 h. Peptides were separated by electrophoresis as described by Pierpoint *et al.* (1977). When peptide 'maps' were made, the paper was sewn on to a piece of 3 MM paper, and chromatographed in

butanol-acetic acid-water-pyridine. The dried paper was inspected under a u.v. lamp (Camag TL 900) or sprayed with conventional ninhydrin sprays (Smith, 1969).

Acid-hydrolysis of proteins and amino acid determinations. Protein (usually 10 mg) was hydrolysed in 6 N-HCl as described by Boulter (1966). Samples were analysed for amino acids by Miss M. Byers, using a Technicon single column analyser with a standard gradient. Samples were also examined for pyridoxyl derivatives by paper chromatography on Whatman 3 MM paper in butanol-acetic acid-water-pyridine (30: 6: 24:20; Fischer *et al.* 1963) or water-methanol-ethanol-benzene-pyridine-dioxane (25:25:10: 10:10: 10; Dempsey & Christiansen, 1962), and also examined by electrophoresis on 3 MM paper at pH 1·9 (1·76 % formic acid; 4 kV for 60 min) and pH 3·5 (pyridine-acetic acid-water; 1:10:89; 2·4 kV for 50 min) in a Shandon HVE apparatus.

Preparation of N-ε-pyridoxyl-L-lysine. N-ε-pyridoxyl-lysine was prepared from poly-L-lysine, by a method similar to that of Schnackerz & Noltmann (1971), and its identity confirmed by its fluorescence, behaviour on paper chromatography (Anderson, Anderson & Churchlich, 1966) and in the Technicon amino acid analyser (Fischer *et al.* 1963). The preparation contained some unmodified lysine (about 30 %) and an unknown, ninhydrin-positive material (about 18 %) which had little or no absorption in the u.v. The u.v. spectrum of the preparation was assumed to be that of N-ε-pyridoxyl-lysine unaffected by these two contaminants.

Spectral measurements. Absorption and fluorescent spectra were measured as previously described (Pierpoint, 1973). The tryptophan fluorescence of virus derivatives was excited at 295 nm, and pyridoxyl-fluorescence at 325 nm; the resulting emission maxima were near 340 and 400 nm respectively. For quantitative comparison, virus or protein solutions were adjusted to have equal absorption at 265 or 290 nm, and to have $E < 0·1$ at the wavelength of excitation. Under these conditions fluorescence was proportional to protein concentration. The presence of SDS (0·5 to 1·0 %, w/v), occasionally used with preparations of limited solubility, had no effect on either absorption or fluorescence spectra of soluble preparations.

Radioactivity. ^{3}H-sodium borohydride, 490 mCi/mmol, was obtained from the Radio-chemical Centre, Amersham. ^{3}H radioactivity was measured on a Beckman LS 250 Liquid Scintillation System. Samples were diluted to 0·5 ml with water and mixed in vials with 5 ml scintillation fluid (8 g butyl-PBD, 0·5 g Ciba scintillator PBBO and 1 l toluene). Corrections for quenching and background radioactivity were made.

RESULTS

Initial reaction of PVX and PLP

When a suspension of PVX (2·7 mg; 0·1 µmol of protein subunits) was mixed at pH 7 with a 24-fold excess of PLP (approx. 1 mM), a small ($E = 0·06$) absorption peak developed at 420 nm. Virus re-isolated after incubation with a larger amount of PLP (20 mM; 210-fold molar excess) and reduction with sodium borohydride, had an absorption peak at 325 nm and was visibly fluorescent under an u.v. lamp. However, viru sseparated from this reaction mixture before being reduced with sodium borohydride, had a spectrum indistinguishable from that of unmodified PVX and did not fluoresce.

The results suggest that the initial reaction between PVX and PLP is the reversible formation of a Schiff base and that this can be reduced to produce a stable pyridoxamine-type derivative such as protein-bound N-ε-pyridoxyl-lysine.

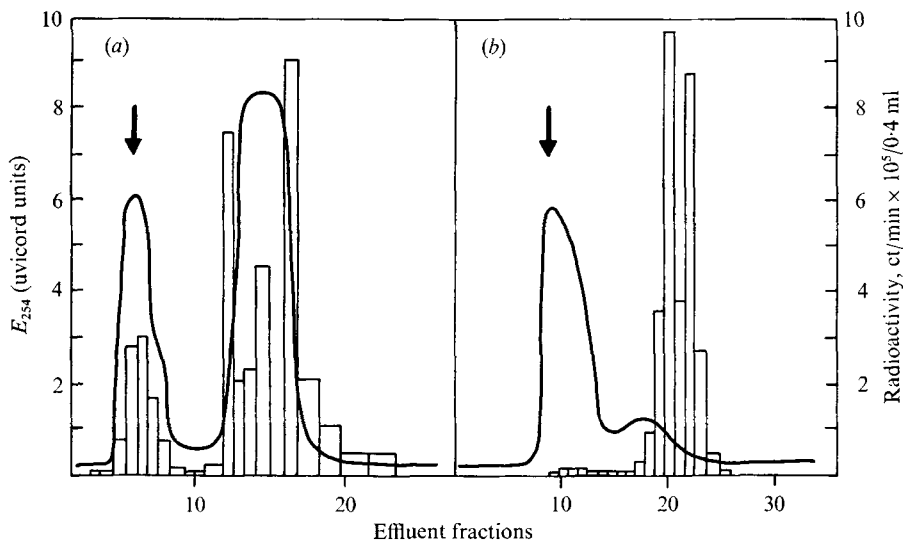Fig. 1. Formation of ^{3}H-labelled PVX-PLP. (*a*) PVX (7 mg) was incubated with 40 μmol of PLP, reduced with a few grains of ^{3}H-sodium borohydride, and excess borohydride removed by acid treatment and de-gassing. The reaction mixture was chromatographed on a column of CPG-10, the effluent monitored at 254 nm (—) and samples (0·4 ml) taken for radioactive counting. PVX-PLP is the first peak of material to emerge from the column (↓). (*b*) PVX (↓) was treated in the same way, but without PLP.

Identification of N-ε-pyridoxyl-lysine in hydrolysates of PVX-PLP

Acid-hydrolysates of PVX-protein contain at least seven substances which can be separated by paper chromatography or electrophoresis and which fluoresce in u.v. light: they are probably derived from the breakdown of tryptophan and tyrosine (Ledvina & Labella, 1971). Hydrolysates of the protein of PVX-PLP contain the same fluorescent substances, and in addition, a blue-fluorescent material which had the same R_f as the major component of the synthetic N-ε-pyridoxyl-lysine on chromatography in butanol-acetic acid-water-pyridine, and electrophoresis at pH 1·9 and 3·5. When eluted after chromatography it had the same fluorescence spectrum as the synthetic material (λ max. excitation = 325 nm; λ max. emission = 395 nm), and co-chromatographed with it in butanol-acetic acid-water-pyridine. Both substances were convincingly separated from reduced derivatives of PLP such as pyridoxine and pyridoxamine, during the chromatography.

An acid-hydrolysate of PVX-PLP protein examined in the amino acid analyser, contained a small amount of ninhydrin-reacting material which was eluted in the same region as synthetic N-ε-pyridoxyl-lysine. It corresponded, assuming it to have a norleucine equivalent of 1, to about 5 % of the amount of lysine in the hydrolysate. This hydrolysate contained about 10 % less lysine than did a comparable hydrolysate of PVX-protein: but as recovery of some other amino acids was low, this decrease must be interpreted cautiously.

Incorporation of ^{3}H-borohydride into PVX-PLP

When PVX which had been incubated with a 200 molar excess of PLP was reduced with ^{3}H-sodium borohydride, the separated virus was, as expected, radioactive (Fig. 1). Its specific activity was only a tenth of the nominal specific activity of the borohydride, partly due to the small amount of PLP bound in this experiment (0·4 molecules/protein
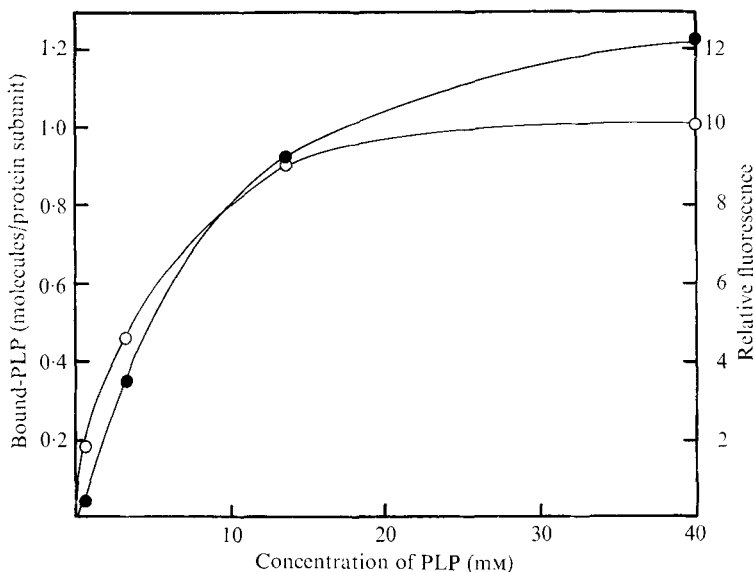
Fig. 2. Effect of PLP concentration on its binding to PVX particles. The particles (3 mg; 0·11 μmol of protein subunits) were incubated in 1·5 ml of phosphate buffer (0·05 M; pH 7·5) containing different amounts of PLP. After incubation at 25 °C for 30 min, all samples were reduced with similar amounts of borohydride, dialysed overnight, and the PVX-PLP separated on a column of CPG-10. Bound PLP was estimated from u.v. absorption spectra (●—●) and also from fluorescence excited at 325 nm (○—○).

subunit). The bulk of the incorporated radioactivity (96·5 %) was recovered in the protein extracted from the virus by acetic acid: only small amounts were detected in the unwashed pellets of nucleic acid (2·8 %) or undegraded virus (1·4 %). Only small amounts of radio-activity were detected in PVX reduced with ^{3}H-borohydride in the absence of PLP (Fig. 1).

Factors affecting the amount of PLP bound to PVX

The amount of PLP bound per protein subunit of PVX depended to a small extent on the time for which the reaction mixture was incubated before reduction, on the pH of the medium and, more markedly, on the concentration of PLP. Decreasing the period of incubation from 30 to 10 min decreased the binding of PLP by 10 %. Lowering the pH from 7·5 to 7·0 brought about a similar decrease (8 %) while raising it to 8 caused an increase (8 to 16 %). Less PLP was bound, judged either by absorption or fluorescence spectra, when the concentration of PLP used was less than 15 mM (Fig. 2). The amount of PLP bound was not increased when tris buffer replaced phosphate (cf Benesch et al. 1973). There was little or no binding at either pH 7 or 6·5 when reduction was effected with sodium cyanohydridoborate (Borch, Bernstein & Durst, 1971).

The amount of PLP bound under standard conditions varied from one preparation to another. Most preparations contained between 0·6 and 1·2 molecules of PLP per subunit, although very occasionally higher values of 1·6 to 1·8 were obtained. A preparation which was pyridoxylated three times with PLP at concentrations of 29 mM, 22 mM and 22 mM and sedimented between each treatment, contained 1·2 to 1·3 molecules PLP per subunit and was as infective as the control sample of unmodified PVX.

Pyridoxal was bound to PVX to about a third of the extent of PLP in the same conditions.

Table 1. *Competition between PLP and chlorogenoquinone for reaction with PVX**

| | | | PLP bound (%) | |
| | | Chlorogenic acid bound | Estimated by | Estimated by |
Expt.	Virus	(μmol/protein subunit)	^{3}H incorporation	fluorescence
1	PVX	0·924 (100 %)	—	—
	PVX-PLP	0·233 (25 %)	—	—
2	PVX	—	100	—
	PVX-Q_1	—	34	—
3	PVX	—	—	100
	PVX-Q_1	—	—	35
4	PVX	—	—	100
	PVX-Q_1	—	—	17

* PVX and PVX-PLP were reacted with chlorogenoquinone as described in Methods; the products were separated by chromatography and their bound chlorogenoquinone estimated as quinic acid (Expt. 1). PVX and PVX-Q_1 were reacted with PLP in the presence of oxidized chlorogenic acid, reduced, purified by chromatography, and their relative content of bound PLP estimated by fluorescence (Expt. 3 and 4): alternatively, PVX-Q_1 was purified after its preparation, and, along with a control sample of PVX, reacted with PLP, reduced with ^{3}H-sodium borohydride, collected by centrifugation and its radioactivity measured (Expt. 2).

This may indicate a partial requirement for the anionic phosphate of PLP (Means & Feeney, 1971), or more probably, be caused by the decreased availability of the aldehyde group of pyridoxal due to internal hemi-acetal formation (Cordes & Jencks, 1962). Pyridoxamine was not, apparently, bound to PVX at all.

Sites of reaction of PLP

Two of the amino groups of PVX subunits are known to be reactive or to lie near the surface of the intact virus. One of them reacts predominantly with chlorogenoquinone, and the other is adjacent to a peptide bond which is readily split by brief exposure to trypsin (Pierpoint *et al.* 1977). Attempts were made to see if PLP modifies either of these groups (1) by reacting PVX-PLP with chlorogenoquinone and by reacting PVX-Q_1 with PLP, (2) by observing the number of fluorescent peptides in tryptic digests of protein stripped from PVX-PLP, and (3) by examining the susceptibility of PVX-PLP to brief exposure to trypsin.

Reactions of PVX-PLP and PVX-Q_1

PVX-PLP reacts with chlorogenoquinone although the amount of quinic acid bound is only a quarter of that bound by unmodified PVX under the same conditions (Table 1). PVX-Q_1 is also apparently less reactive to PLP than is PVX (Table 1). Both the methods of estimating the PLP bound to PVX-Q_1 are open to criticism: thus the fluorescence of PLP bound to PVX-Q_1 may be more quenched than that bound to PVX, and the radio-activity incorporated from ^{3}H-borohydride by PVX-Q_1 needed to be corrected for a large amount of radioactivity incorporated into PVX-Q_1 in the absence of PLP. Nevertheless both estimates agree in suggesting that PVX-Q_1 binds between 1/5 and 1/3 as much PLP as does PVX.

There is therefore, appreciable, but not complete, competition between chlorogeno-quinone and PLP for reaction sites on PVX. The competition suggests that both reagents react with the same amino group, or, less likely, that once reacted at different sites they
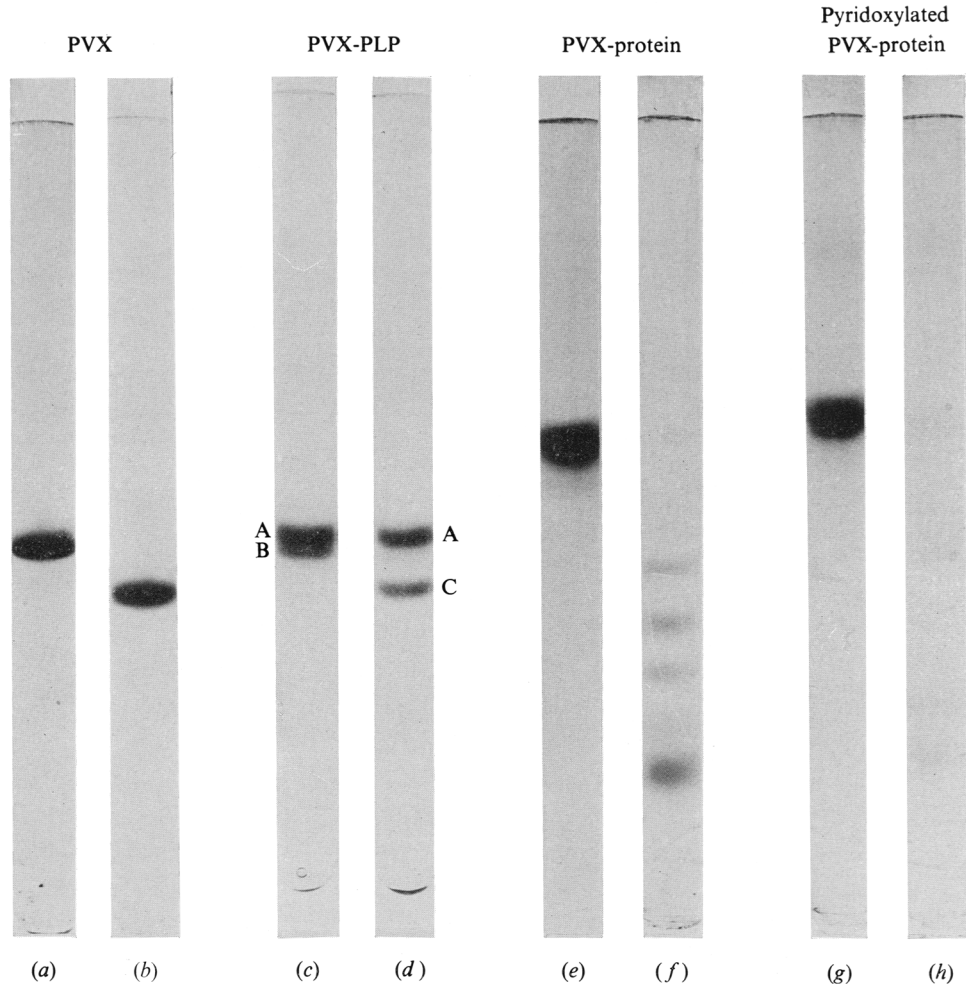
Fig. 3. Effect of brief trypsin treatment on PVX, PVX-PLP, PVX-protein and pyridoxylated PVX-protein. The virus particles and proteins were treated with trypsin (ratio 100:1) at 20 °C for 15 min. Control (*a*, *c*, *e*, *g*) and treated (*b*, *d*, *f*, *h*) samples of the resulting polypeptides were analysed in SDS-acrylamide gels containing 5 % (gels *a* to *d*) or 10 % acrylamide (gels *e* to *h*). PVX-PLP contained approx. 1 molecule of PLP per protein subunit, and pyridoxylated PVX-protein, 3·7 molecules per subunit.

induce a conformational change that makes the other site less reactive. But the fact that competition is not complete suggests that one or both reagents react at more than one site.

Fluorescent peptides derived from PVX and PVX-PLP

A comparison of peptide maps obtained from trypsin-digests of PVX-protein and PVX-PLP-protein, also suggested that PLP combines with more than one site on PVX.

Maps derived from PVX-PLP protein were similar to those (unpublished) maps derived from PVX-protein, but contained more material near the origin which tended to streak. The maps suggested that 2 peptides which moved little during electrophoresis at pH 6·5 but more so during chromatography, were either missing or decreased in intensity. They
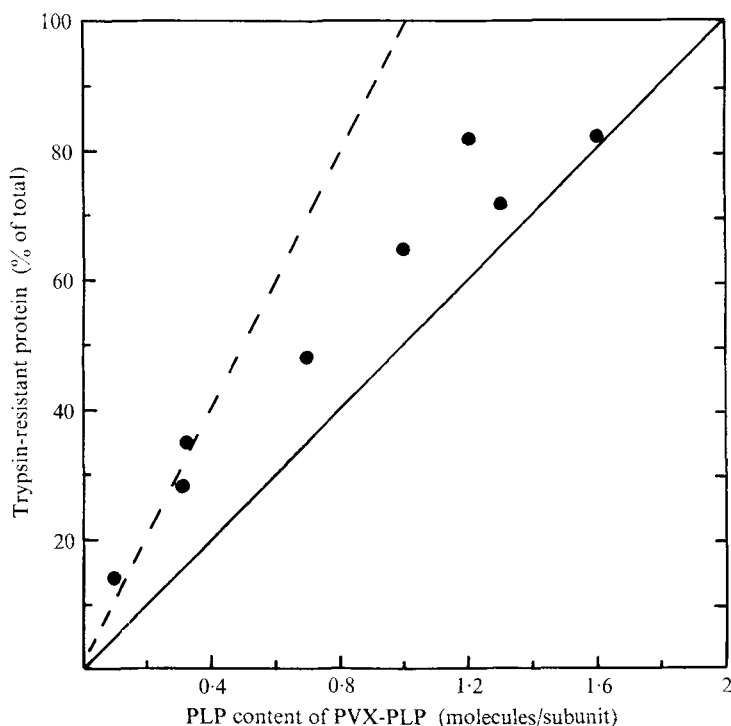
Fig. 4. Proportion of trypsin-resistant protein in PVX-PLP preparations. PVX-PLP preparations were briefly treated with trypsin, their protein analysed in SDS-polyacrylamide gels and the fractions estimated from traces made by scanning the stained gels in a densitometer. The unbroken line indicates the proportion of trypsin-resistant protein expected, assuming that PLP is evenly distributed between two sites only one of which confers trypsin-resistance: the broken line indicates the proportion of trypsin-resistant protein expected if PLP only modifies the site conferring trypsin-resistance.

also showed the presence of two areas of PLP-like fluorescence. One (X) moved slightly as a diffuse spot to the anode at pH 6·5, and the other (Y) as a more discrete spot to the cathode. They were much fainter and less easy to locate after chromatography: X migrated 1/3 the distance of the fastest moving peptide, Y moved slightly further. Both spots were seen in digests of PVX-PLP whose PLP content ranged from 0·5 to 1·4 molecules of PLP per protein subunit. The spots derived from the preparation with the least PLP (0·5 molecules/subunit, as judged from its spectrum) were eluted and shown to contain pyridoxyl-like fluorescence in the ratio 6:1. Judged visually, preparations containing more PLP appeared to have relatively more Y. Control experiments confirmed that neither X nor Y was contaminating PLP, pyridoxal or pyridoxamine.

Effect of brief exposure to trypsin on PVX-PLP

Convincing evidence that PLP combines with at least two sites on PVX was obtained by examining PVX-PLP by electrophoresis in SDS gels both before and after brief exposure to trypsin. PVX itself gives a single protein band in these gels, with only occasional traces of a faster moving species with lower molecular weight; after brief trypsin treatment it is entirely converted to a low molecular weight form (Fig. 3 *a* and *b*; see also Pierpoint *et al.* 1977). Preparations of PVX-PLP judged to contain about 1 molecule of PLP per protein
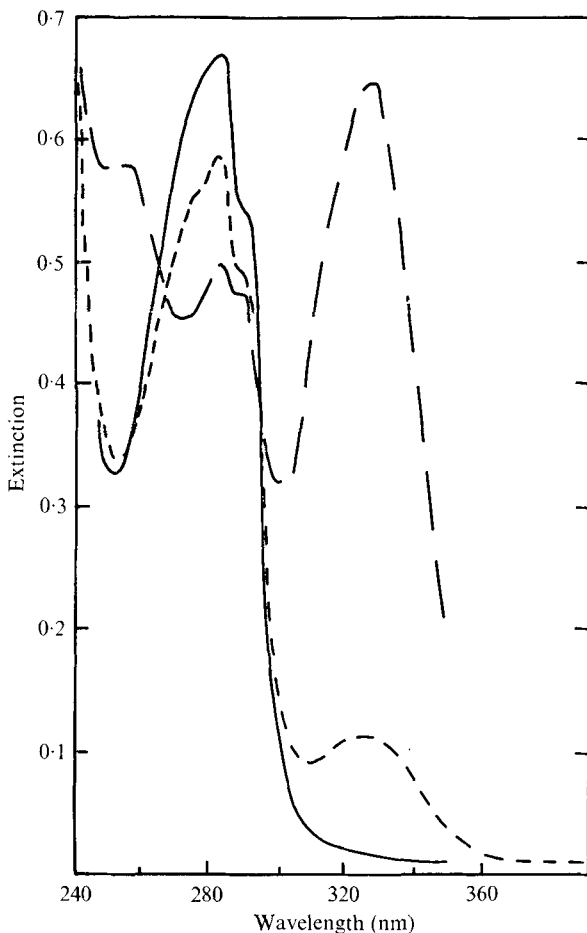
Fig. 5. Absorption spectra of PVX-protein (——), protein stripped from PVX-PLP (---) and pyridoxylated PVX-protein (— —). Solutions containing approx. 110, 95 and 54 nmol of protein per ml respectively, were in phosphate buffer (0·05 M, pH 7), and contained in 2 mm cells. The PVX-PLP from which protein was stripped contained approx. 0·5 molecules PLP/protein subunit.

subunit migrated in the gels as two poorly resolved bands (Fig. 3c); the slower band (A) was unaffected by trypsin, but the other (B) was completely converted to material (C; Fig. 3d) which moved as fast as the low molecular weight material derived from PVX by trypsin. Both A and C fluoresced under an u.v. lamp. When the gels were cut into slices approx 2·5 mm thick, and the slices extracted with water, the clarified solutions containing A and C both showed a fluorescence characteristic of protein-bound pyridoxal derivatives; they had a broad fluorescence band at 400 nm which was maximally excited at 325 to 330 nm and had a smaller excitation peak at 295 nm. Solutions derived from gel slices between A and C had less fluorescence. In the experiment illustrated in Fig. 3(d), the proportion of material in A and C was 2:1 as judged by scanning the stained gels, 3:1 by comparing the extracted fluorescence that could be excited at 325 nm, and 2:1 by comparing extracted fluorescence excited at 295 nm.

The protein in band A has probably been rendered resistant to trypsin by the reaction of PLP at the ε-amino group that is recognized by trypsin (see Pierpoint et al. 1977). The

Table 2. *Amino acid content of PVX-protein before and after pyridoxylation**

	PVX-protein		Pyridoxylated PVX-protein	
	μmol/10 μl hydrolysate	Ratio (val = 13)	μmol/10 μl hydrolysate	Ratio (val = 13)
Asp	0·0416	22·4	0·0285	21·9
Thr	+ + +		0·0370	28·5
Ser	0·0274	14·7	0·0180	13·9
Glu	0·0315	16·9	0·0213	16·4
Pro	0·0246	13·2	0·0180	13·8
Gly	0·0225	12·1	0·0156	12·0
Ala	+ + +		0·0473	36·4
Val	0·0242	13·0	0·0169	13·0
Cys/2	0·0022	1·1	0·0029	2·3
Met	0·0122	6·6	0·0089	6·9
Ileu	0·0211	11·4	0·0143	11·0
Leu	0·0170	9·1	0·0119	9·1
Tyr	0·0041	2·2	0·0026	2·0
Phe	0·0207	11·1	0·0145	11·1
Lys	0·0192	10·3	0·0074	5·7
His	0·0037	2·0	0·0027	2·2
Pyridoxyl-Lys	0	0	0·0060	4·6
Arg	0·0174	9·4	0·0126	9·7

* A sample of pyridoxylated PVX-protein, whose spectrum suggested that it contained 4·2 molecules of PLP per protein subunit, was hydrolysed in 6 N-HCl at 110 °C for 20 h and analysed for amino acids. A sample of PVX-protein from which it was made was treated in the same way but contained too much Thr and Ala to be estimated (+ + +). The ratio of amino acids in the two hydrolysates were compared by adjusting to valine values of 13 (columns 2 and 4) which is assumed to be the number of molecules of valine released from each protein subunit by 20 h hydrolysis.

trypsin-sensitive protein in band B must have PLP bound at another amino group, probably the one that reacts with chlorogenoquinone. The proportions of A and C derived from preparations of PVX-PLP, containing about 1 PLP molecule per protein subunit, suggest that PLP combines more readily with the first site than the second. This conclusion also follows from comparing the proportions of A and C in virus containing different amounts of PLP. Unmodified protein, like the protein in B, is trypsin-sensitive, so that if both amino groups were equally available to PLP, a graph of the % of trypsin-resistant material in PVX-PLP against the amount of bound PLP should be a straight line passing through the origin. The observed points, however, deviate from this (Fig. 4), suggesting that preparations, especially those containing little bound-PLP, are more trypsin-resistant than predicted. Subject to the caution that there are large errors in measuring small amounts of protein-bound PLP, it is therefore likely that the trypsin-recognition site on PVX is more readily modified by PLP.

The partial separation by electrophoresis in SDS-containing gels of two forms of PVX-PLP which probably differ only in the site of substitution, suggests that properties other than molecular weight influence electrophoretic movement in the conditions used.

Reaction of PLP with isolated PVX-protein

Protein stripped from PVX and freeze-dried, reacts more extensively with PLP than does the protein of intact virus. The u.v. spectrum of the preparation shown in Fig. 5 has a peak at 325 nm whose size suggests that it contains 5·3 molecules of PLP per molecule of protein. Its fluorescence at 400 nm was about eight times that of a preparation of protein stripped from PVX-PLP that contained 0·5 molecules of PLP per subunit (Fig. 6b). The
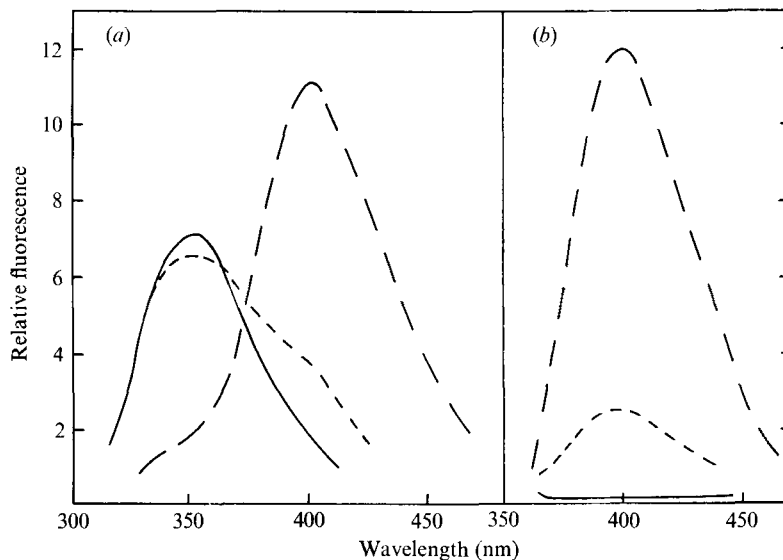
Fig. 6. Fluorescence spectra of PVX-protein (——), protein stripped from PVX-PLP (– – –) and pyridoxylated PVX-protein (— —). (a) Excited at 290 nm; instrument gains setting at 1K. (b) Excited at 235 nm; gains setting 100, fine gains 7. The three proteins were dissolved in phosphate buffer, pH 7, 0·05 M, so that E_{290} was 0·1. SDS (0·5 %) was also present in the solution of protein stripped from PVX-PLP.

pyridoxal-type fluorescence was predominant when excitation was at wavelengths (290 to 295 nm) which produce the 'tryptophan' fluorescence of PVX (Fig. 6a; Pierpoint, 1973; Homer & Goodman, 1975) and fluorescence at 340 nm was relatively decreased, suggesting an energy transfer from the tryptophan to bound-PLP. Other preparations of the modified protein contained 3·9 to 5·8 PLP molecules/subunit, although one made from a protein that had a limited solubility contained 1·2 molecules. Extensive substitution is not a consequence of changes that occur during freeze-drying; thus an undried preparation reacted with 4·8 molar equivalents of PLP.

A preparation of pyridoxylated protein judged to contain 4·2 molecules of PLP per molecule of protein, was hydrolysed and examined in the amino acid analyser. It contained a substance which eluted as N-ε-pyridoxyl-lysine, and which, assuming that it had a nor-leucine equivalent of 1, corresponded to 4·6 molecules per molecule of protein (Table 2). The hydrolysate contained only 55 % of the lysine recovered from a comparable hydro-lysate of PVX-protein, suggesting the loss of 4·9 to 5·4 lysine residues from each protein molecule; all the other amino acids were recovered to a similar extent as those from PVX-protein.

Pyridoxylated PVX-protein is extensively broken down on brief exposure to trypsin. Electrophoretic analysis of the reaction in SDS-acrylamide gels showed that the break-down resembles the degradation of PVX-protein but went more rapidly (Fig. 3e to h). After exhaustive trypsin-digestion (ratio of trypsin to protein 1:25; digestion for 24 h), paper electrophoresis revealed 2 novel fluorescent peptides which migrated to the anode as well as poorly resolved fluorescent substances of opposite charge.

DISCUSSION

The amount of PLP bound by different preparations of PVX when treated under similar conditions may vary by as much as 20 to 30 %. This variation may partly reflect difficulties in the estimation of bound PLP, although we think that these are only appreciable when preparations contain little bound PLP and scatter a lot of light. The major cause of the variation is likely to be small differences in the conditions of the reduction of the Schiff base of PLP and PVX with borohydride. Borate is formed in this process (Gaylord, 1956) and causes an alkaline drift which was neutralized manually. Differences in transient pH changes before and during neutralization may affect the reactivity of the amino groups of PVX; a preparation, whose pH was inadvertently allowed to rise above 8 during the reduction and which was tardily neutralized, was resolved by electrophoresis into many protein bands after a brief exposure to trypsin. It has been shown by Dempsey & Christiansen (1962) that the pH at which albumin-PLP adducts are reduced determines the site on the protein at which PLP is bound.

It has previously been argued that PLP reacts predominantly with the ϵ-amino groups of PVX-protein (Pierpoint, 1974). This is now confirmed by the demonstration of N-ϵ-pyridoxyl-lysine in hydrolysates of PVX-PLP. Alternative reactions involving the terminal α-amino group of the protein (e.g. Benesch *et al.* 1973) or aldoxime structures involving thiol groups (Fischer *et al.* 1963) are very unlikely, because these groups are either blocked or unreactive in PVX-protein (our unpublished information). However, although the present results confirm the limited reaction of PLP with PVX, they make it unlikely that reaction involves only one specific ϵ-amino group (Pierpoint, 1974). PVX-Q_1 reacts with PLP to a limited extent; moreover PVX-PLP, estimated to contain one or less PLP molecules bound to each protein subunit, contains two types of PLP-fluorescent subunit, and digestion by trypsin produces two fluorescent peptides. These results are more simply explained by the partial, simultaneous reaction with PLP of the two ϵ-amino groups that have previously been characterized. From the evidence summarized in Fig. 4, the amino group adjacent to the trypsin-sensitive site is the more extensively pyridoxylated of the two. The limited reaction of the two amino groups with PLP could conceivably be due to an interaction, so that reaction of one of them hindered reaction of the other.

Many proteins bind PLP specifically at or near phosphate-binding sites (Glazer, 1976). It is not this selectivity which restricts the binding of PLP to two of the 11 to 12 ϵ-amino groups in each subunit of intact PVX, for PLP reacts more extensively with isolated but undenatured protein subunits. Glazer (1976) discusses other factors which limit the reactivity of functional groups in proteins either by affecting their pK_a or imposing a steric hindrance to their reactivity. It is not known which factor operates in PVX but it may be germane that Goodman (1977) has recently demonstrated, by the fluorescence of a bound dye, that there are hydrophobic regions in intact PVX which do not occur in isolated subunits. The proximity of some amino groups to these regions in intact PVX might account for their inability to react with PLP. However, whatever makes the amino groups in PVX unavailable to PLP, and also to chlorogenoquinone, does not appear to restrict their availability to TNBS and MP (Pierpoint, 1974).

Finally, it is notable that two modifications to PVX which could conceivably occur *in vivo*, reaction with enzymically generated *o*-quinones and loss of the N terminal peptide by a trypsin-like protease, do not inactivate the virus. The two most accessible amino groups in the virus are thus inessential to infectivity while, apparently, more essential groups are less available. This property is obviously a consequence of the primary structure

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of PVX-protein, and it may only be a coincidental one; however, it may be a character which confers some protection on PVX *in vivo*, and which has been the subject of evolutionary selection.

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