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MODIFICATION OF PROTEINS DURING THE OXIDATION OF LEAF PHENOLS: REACTION OF POTATO VIRUS X WITH CHLOROGENOQUINONE

WILLIAM S. PIERPOINT, ROBERT J. IRELAND and JOHN M. CARPENTER

Departments of Biochemistry and Plant Pathology, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, U.K.

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Key Word Index—Potato virus X; ε-amino groups; chemical modification; polyphenols; chlorogenic acid.

Abstract—Potato virus X (PVX) is modified by incubation with chlorogenic acid and polyphenoloxidase. The product made at pH 7 (PVX-Q_1) is grey in colour, retains about 2/3 of its initial infectivity, and contains, on average, 1 molecule of bound chlorogenic acid per protein subunit. The product made at pH 7.8 (PVX-Q_2) is blue, retains at least $\frac{1}{3}$ of its infectivity, and contains approximately 2 molecules of chlorogenic acid per subunit. Both preparations contain a proportion (18–42%) of cross linked subunits. Brief exposure to trypsin converts subunits of both types of PVX-Q to a form with a slightly lower MW; the reaction goes more extensively with PVX-Q_1 (80% converted) than with PVX-Q_2 (45% converted). Prolonged exposure to trypsin degrades both forms of PVX-Q to free quinic acid and peptides, apparently only one of which contains chlorogenic acid. It is argued that, in PVX-Q_1, predominantly one specific lysine ε-NH_2 has been modified with chlorogenoquinone. The structure of PVX-Q_2 is less clear.

INTRODUCTION

When potato virus X is incubated with chlorogenic acid (CA) and polyphenoloxidase (PPO), it is chemically modified but not inactivated [1]. The modification was interpreted as a reaction between the enzymically generated *o*-quinone, chlorogenoquinone, and the ε-amino groups of some of the lysine residues in the protein subunits of the virus: the formation of chlorogenoquinone has been demonstrated by trapping procedures [2, 3], and the presence of "non-essential" amino groups on PVX demonstrated by reaction with specific reagents [4]. Although it was supposed, on the basis of known reactions, that the lysine ε-N was substituted into the quinone ring of chlorogenoquinone, there was no direct evidence on the nature and number of these linkages. It was thought worthwhile to obtain such evidence because of the possibility that quinone-modified virus (PVX-Q) may exist in infected senescing leaves and contribute to their infectivity [1], and also because reactions between plant proteins and oxidizing phenols occur in many natural and food processing systems [5, 6], and little is known of their mechanisms.

PVX is a flexuous thread approx. 515 nm long, 13 nm wide, and containing 6% RNA [7]. The protein consists of about 1400 helically arranged, probably identical, subunits. The subunits of the strain of virus used in the present work, have a MW near 27,000 d and contain 12–14 lysine residues (unpublished results). In this work we estimate the amount of CA bound in PVX-Q, by measuring the quinic acid that is released from it on saponification. We also present evidence on crosslinks between protein subunits in PVX-Q, and the degree to which the reaction with quinone involves a specific lysine residue in PVX which has been characterized by its susceptibility to trypsin [8, 9].

RESULTS

Chlorogenic acid content of PVX-Q

PVX-Q, chromatographically separated from polymerized CA and PPO gives, after overnight saponification, a pink colour with periodate-thiobarbituric acid. The colour has a spectrum (λ_{max} 549 nm) identical with that produced by authentic quinic acid, indicating that saponification releases the quinic acid moiety of bound CA. No colour is produced from unmodified PVX. No extra colour is produced by recovering the saponified PVX-Q and re-saponifying it overnight. A single saponification is also sufficient to liberate quinic acid quantitatively from chlorogenic acid.

The amount of quinic acid recovered from PVX-Q has been used to estimate the amount of chlorogenic acid bound per protein subunit (Table 1). Preparations made at pH 7 (PVX-Q_1), and which retained at least 2/3 of their infectivity as measured on *Chenopodium amaranticolor*, contain approx. 1 molecule of CA per subunit. Preparations made at pH 7.8 (PVX-Q_2) contain twice as much CA and are generally less infective: those made at pH 6.0 contain about 0.8 molecules of CA per subunit, and retain virtually all their infectivity. The colour and the UV spectra of preparations depends on the pH at which they are made (Table 1; Fig. 1); those made at pH 7.8 are blue enough for their movement down porous glass columns to be followed visually. The higher content of CA in PVX-Q made at pH 7.8, is not due to the oxidation of more CA at this pH than at pH 7. Reaction mixtures at both these pH's, showed, after the incubation, little or no oxygen absorption: oxygen absorption ensued in both when more CA was added, demonstrating the presence of excess, active oxidase.

PVX-Q_1 is not resolved into nucleic acid and protein by treatment with cold glacial acetic acid as readily as is

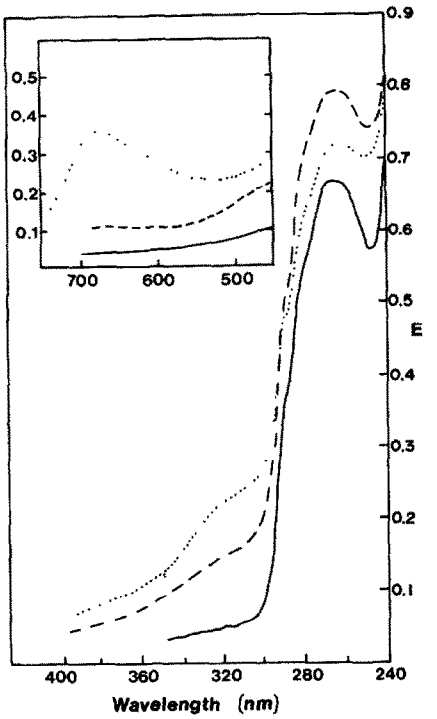


Fig. 1. Spectra of PVX (———), PVX-Q_1 (– – – –) and PVX-Q_2 (·····). Solutions had similar, but not identical amounts of virus (approx. 400 μg/ml in phosphate buffer, 0.05 M, pH 7) and were contained in 5 mm spectrophotometer cells. Inset is the visible spectra of the solutions contained in 40 mm cells.

PVX. Although nucleic acid from PVX-Q_1 has not been separated free enough from protein to determine if it contains any bound CA, evidence suggests that the bulk of the bound CA is attached to protein: a sample of protein from PVX-Q_1, whose P content, and hence whose nucleic acid content, was less than a tenth of that of PVX-Q_1, contain as much bound CA as did PVX-Q_1 (Table 1).

Cross linking of subunits in PVX-Q

The proteins of PVX and PVX-Q were examined by electrophoresis in polyacrylamide gels (5%) containing SDS. The protein subunits of PVX migrated as a single species (Fig. 2a), although preparations occasionally contained a small amount of a faster moving component that is probably the result of the removal of a single peptide by a trypsin-like plant protease [8–10]. Most of the protein of PVX-Q migrated to the position of unmodified subunits, but there were also slower moving bands at positions expected of subunit dimers, trimers,

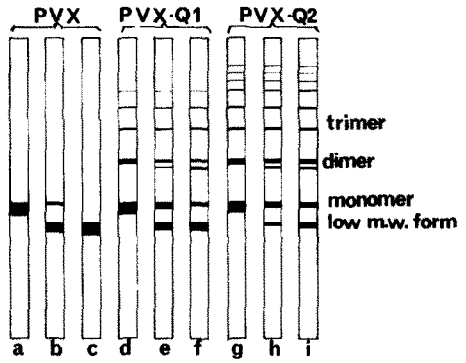


Fig. 2. Electrophoresis of PVX and PVX-Q in SDS-gels, before and after brief treatment with trypsin. Gels were loaded with 16 μg of virus, and the intensity of the protein bands approximately indicated by their width. Virus samples (a) PVX; (d) PVX-Q_1; (g) PVX-Q_2. Virus after exposure to trypsin (100:1) for 5 min; (b) PVX, (e) PVX-Q_1; (h) PVX-Q_2; virus after exposure for 30 min; (c) PVX; (f) PVX-Q_1; (i) PVX-Q_2

and polymers up to octamers (Figs 2d and g). Thus, modifying PVX with chlorogenoquinone, cross-links an appreciable proportion of the subunits, but does not lead to degradation.

Quantitative scanning of the gels shown in Fig. 2 indicates that more cross-linking occurs in PVX-Q_2 than in PVX-Q_1 (Table 2): less than 10% of the subunits were crosslinked in PVX-Q made at pH 6.

Table 1. Chlorogenic acid-content, infectivity and colour of PVX-Q preparations

Preparation	pH at formation	Colour of dried virus	Infectivity (% of unmodified PVX)	Chlorogenic acid (molecules/subunit)
(1) PVX-Q_1	7.0	grey	—	1.00
(3) PVX-Q_1	7.0	grey	62	0.95
(5) PVX-Q	6.0	red–brown	105	0.77
(6) PVX-Q_2	7.8	blue	56	1.97
(8) PVX-Q_2	7.8	blue	36	1.95
PVX-Q_1-protein	7.0	—	—	1.00

Table 2. Polymer composition of the protein subunits of PVX-Q

	Polymer (approx % of total protein)							
Preparation	subunit	dimer	trimer	tetramer	pentamer	hexamer	heptamer	octamer
PVX-Q_1	82	12	4	1	0.5	—	—	—
PVX-Q_2	58	22	11	5	2.5	1	0.5	0.2

The values are based on the results of scanning 10 gels of each type of preparation. The proportion of protein as monomer varied between preparations by ±5%.

Partial degradation of PVX-Q by brief exposure to trypsin

The peptide bond in PVX which is split by plant proteases or trypsin to produce the low MW form of the protein [8–10], involves the carboxyl group of a lysine residue which must lie on or near the surface of the protein. Modification of this exposed lysine by chlorogenoquinone, like chemical modification of lysine ε-amino groups in other proteins [11], would be expected to prevent this hydrolysis by trypsin. A comparison of the action of trypsin on PVX and PVX-Q would therefore indicate if this lysine was modified in PVX-Q.

Electrophoretic examination in SDS-containing gels showed that a 10 min treatment with trypsin completely converted the subunits of PVX to a low MW form (Fig. 2c). The protein of PVX-Q was converted more slowly and less completely (Figs 2f, 2i; Table 3); even after 90 min, about 20% of PVX-Q_1 protein, and 55% of PVX-Q_2 protein remained unconverted. Table 3 illustrates the time course of the conversion of monomeric and dimeric subunits of both forms of PVX-Q, and shows that the dimers are about twice as resistant to trypsin as monomers. It proved difficult to quantify the conversion of higher polymers, but they are likely to be at least as resistant as dimers.

These results suggest that at least 80% of the trypsin-sensitive lysine residues in PVX-Q_1 and 45% of those in PVX-Q_2 are not modified by quinone. They also suggest that between half (PVX-Q_1) and 1/4 (PVX-Q_2) of the crosslinks in the dimers do not involve the trypsin-sensitive residues. All these values are probably underestimates: some trypsin-sensitive residues may well have been rendered resistant, not by direct modification, but because an adjacent modification restricts their accessibility to trypsin.

Quinic acid-containing peptides in tryptic digests of PVX

If the CA which is bound in PVX-Q_1 is primarily attached to one specific lysine residue, and if this modified lysine is resistant to hydrolysis by trypsin, a peptide map of trypsin-digested PVX-Q should differ from one of unmodified PVX; two 'normal' peptides should be missing, and a novel one present. Preliminary attempts to demonstrate this difference (P. C. Jewer and W. S. Pierpoint, unpublished) have not been successful. However, the presence of one peptide containing quinic acid could be demonstrated in electrophoretograms of trypsin digests of freeze-dried PVX-Q_1.

The quinic acid in such digests did not move appreciably from the point of application during electrophoresis at pH 1.9. At pH 6.5 however, the bulk of it moved towards the anode as two substances (Fig. 3). The slower material was probably peptide bound-quinic acid as it reacted with periodate-barbiturate only after saponification. This area of the electrophoretogram stains with ninhydrin, but is known to contain at least 3 "normal" PVX-peptides (Carpenter; Jewer; unpublished). The faster substance was free quinic acid; it migrated the same distance as authentic quinic acid, and reacted with periodate–thiobarbiturate without saponification. It is, presumably, liberated from the peptide in the course of the trypsin-digestion. The small amount of bound quinic acid that remained at the origin on the electrophoretograms was probably derived from insoluble or partly digested protein that was incompletely removed before application to the paper. A dark coloured material that moved towards the cathode contained little or no quinic acid; it may correspond to the quinic-containing, anode-migrating material that has lost both its quinic acid and its net negative charge during the digestion. Chromatographing these tryptic digests of PVX-Q_1, in butanol–pyridine–acetic acid–water, confirmed the presence of free quinic acid (R_f 0.26); all the bound quinic remaining near the point of application.

Although a more complicated distribution of bound quinic acid was expected in electrophoretograms of digests of PVX-Q_2, the observed distribution was virtually identical to that from PVX-Q_1. The bulk of the bound quinic acid moved towards the anode as if it were a single substance, and the ratio of bound to free quinic acid was very close to the ratio observed with digests of PVX-Q_1. If there are any additional quinic acid-peptides in these extracts they must migrate at the same speed as the peptide (s) in digests of PVX-Q_1, and break down to free quinic acid to the same extent. There is no evidence for a major quinic-containing peptide in the insoluble fraction of the digests: the proportion of quinic acid that was insoluble after tryptic digestion, was approximately 20% in digests of both PVX-Q_1 and PVX-Q_2.

Table 3. Partial degradation of PVX and PVX-Q by trypsin

Time of exposure (min)	Protein converted to low MW form (% of total protein in the gel)				
	PVX monomer	PVX-Q_1 monomer	PVX-Q_1 dimer	PVX-Q_2 monomer	PVX-Q_2 dimer
0.33	14	8	1	9	1
1	30	19	2	18	3
5	90	50	4	27	4
10	100	63	6	30	5
30–90	—	69	6	30	6

Maximum amounts of low MW forms were produced within 30 min incubation. Incubation beyond 90 min produced more extensive degradation at sites other than the sensitive lysine.

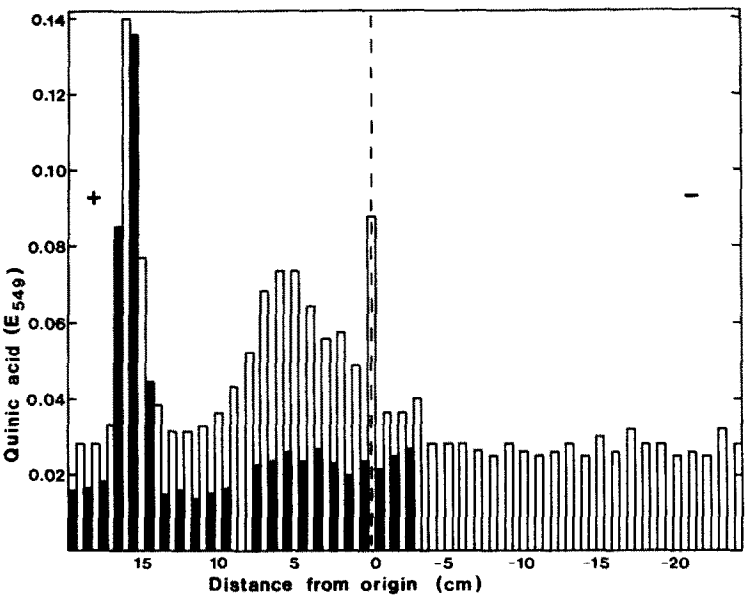
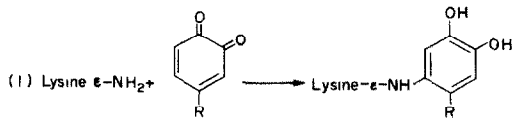


Fig. 3. Distribution of quinic acid in electrophoretograms of trypsin-digested PVX-Q_1. After electrophoresis of the digest at pH 6.5, the paper was cut into 0.5 cm strips, and alternate strips taken for the estimation of free quinic acid (solid bars) or total quinic acid (open bars).

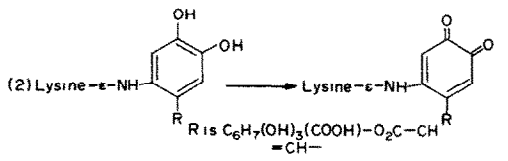
DISCUSSION

Primary reaction of chlorogenoquinone with PVX

Infectivity of PVX is not appreciably diminished when, on average, 0.75 molecules of CA are bound per protein subunit, and is only diminished to a third when 2 molecules are bound (Table 1). This result resembles, and was anticipated from, the modification of PVX with pyridoxyl-5-phosphate, picryl sulphonic acid and methyl picolinimidate [4]. The resemblance argues that chlorogenoquinone reacts with the ε-amino groups of the virus protein, as do the other reagents. The initial reaction is likely to be a substitution of the $-NH_2$ into the quinone nucleus [6, 12], possibly into the 6′ position [3, 13]:



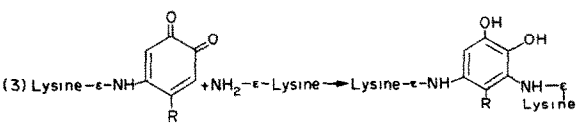
This may be followed by the oxidation of the amino-substituted phenol to quinone by excess chlorogenoquinone:



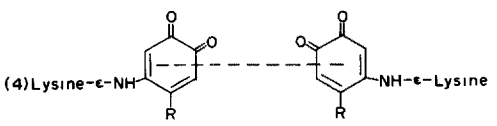
Reaction (1) probably requires an unprotonated amino group, and this may explain why more CA is bound at pH 7 than at pH 6. The second molecule of CA may also be bound because another accessible amino group is deprotonated at pH 7.8. However, there are other possible explanations for this reaction. It is possible, for instance, that the protein conformation changes at pH 7.8 and makes a second $-NH_2$ accessible. It is also possible that at pH 7.8, either one or both molecules of CA react other than by reaction (1), and this would help explain the unexpected blue colour of the product. Blue colours have been observed to form slowly in alkaline solutions containing chlorogenoquinone and substances with free α-NH_2 groups [14]; however there are no free α-NH_2 groups in PVX. Blue compounds are also formed between synthetic chlorogenoquinone and aliphatic amines (Dr. R. Davies, unpublished), although in solvents where chlorogenoquinone reacts differently than in water [3]. The blue and blue-green substances extracted by alkali from CA-rich plant materials [15, 16] are perhaps more relevant to the blue form of PVX-Q, but not enough is known about them to throw any light on the chromophore in PVX-Q_2.

Formation of cross links

The cross links formed between protein subunits indicate that chlorogenoquinone acts as a bifunctional reagent. Two mechanisms can be suggested. In the first, reaction (2) could be followed by a second substitution of a lysine ε-NH_2 into the substituted quinone:



and subsequent oxidation of the bridging nucleus to the quinone state. In the second, two monosubstituted quinone molecules may join by the type of link, possibly an aryl-alkyl ether link [17], that occurs in polymers of caffeyl derivatives.



Whatever their mechanisms, these reactions can only occur between neighbouring lysine residues, and the formation of crosslinks therefore indicates that neighbouring subunits of PVX have pairs of lysine residues in close proximity. Although they have not so far been detected, it is very likely that cross links occur between adjacent lysine amino groups within the same subunit.

Reactive —NH_2 *sites in PVX*

In the preparation of PVX-Q, the protein subunits are exposed to the oxidation of a 100-fold excess of CA. The amount bound per subunit, however, corresponds quite closely to 1 molecule at pH 7 and 2 at pH 7.8. This suggests that bound CA is not spread randomly among the 12–14 amino groups of each subunit, but reacts principally with one specific residue at pH 7 and perhaps 2 at pH 7.8. There is some support for this belief from the reaction of PVX-Q_1 with trypsin. Thus a brief exposure to trypsin produces the low MW form of the protein, indicating that the trypsin-sensitive lysine is predominantly (80%) unmodified. Moreover, more extensive degradation of PVX-Q_1 by trypsin, produces only one detectable quinic-containing peptide. There are some curious and unexplained features of this digestion. It is, for instance, surprising that free quinic acid is produced, and the most likely explanation, that it is produced from the quinic-containing peptide by an esterase activity of trypsin, has yet to be proved. However, in spite of this, it can reasonably be argued that PVX-Q_1 is primarily modified at a particular amino group. The situation is less clear for PVX-Q_2; its trypsin-sensitive site is appreciably modified or otherwise blocked, but, apparently, only one soluble quinic-containing peptide is released on prolonged digestion. From the facts so far available, no certain conclusion can be made about the nature or location of modified groups in PVX-Q_2. But it seems clear that the reaction of chlorogenoquinone with PVX, is, like the reaction of pyridoxal-5-phosphate with PVX, more restricted and more selective than that of some other amino group-modifying reagents [4].

EXPERIMENTAL

Virus production and modification. A necrotic strain of PVX was cultured in, and isolated from glass-house grown tobacco plants (*Nicotiana tabacum*, var. Xanthi nc) as previously described [1]. Its concentration was estimated from its $E_{265\ nm}$, uncorrected for light scattering, by assuming $E^{0\ 1\%}_{1\ cm}$ for this strain to be 3.5 [18]. PVX-Q was prepared essentially as described previously [1], by incubating PVX (10–15 mg in 1.0–1.7 ml phosphate buffer, 50 mM; pH 7) with CA (30 μmol in 0.5 ml) and PPO (0.4–0.5 ml of preparation 1; 0.2 ml of preparation 2) both added in 4–5 successive portions at approx 30 min intervals. For incubation at pH's other than 7, more concentrated buffer, approx 75 mM, was used. Modified virus was separated from low MW material by chromatography on a column (107 × 1.2 cm) of glass granules (CPG-10; pore diameter 1400 Å; BDH) equilibrated with phosphate buffer (50 mM; pH 7). Virus emerged in the void vol (45–46 ml) and samples were taken for infectivity, trypsin-treatment or gel electrophoresis. The rest was dialysed against distilled water for more than 60 hr and freeze-fried. The CPG granules had been pretreated with 1% (w/v) Carbowax 20 M to prevent the absorbtion of virus [19], and pack with the aid of a vibrator. *Virus infectivity* was estimated by the number of local lesions produced on *Chenopodium amaranticolor* [1]. *Polyphenoloxidase* (E.C. 1.10.3.1) was partially purified from Xanthi tobacco as previously described [1]. The two preparations used in the present work oxidized CA at the rates of 2.7 (prep 1) and 5.6 (prep 2) μl O_2 uptake/min/0.01 ml, when measured at 26° in an oxygen-electrode (capacity 3 ml) containing CA (4 mM), benzene sulphinic acid (33 mM) and Tris–HCl buffer (67 mM; pH 7).

Gel electrophoresis of virus protein [20]. 5% Acrylamide gels, also containing 0.133% bis-methylene-acrylamide, 0.1% SDS and 0.05 M sodium phosphate pH 7.1, were prepared in 100 × 5 mm (diameter) glass tubes. Gels were loaded with 16–20 μg protein and a current of 6 mA/gel at 50 V applied for 3½ hr at ambient temp. Gels were stained overnight with 0.01% Coomassie brilliant blue in H_2O–MeOH–HOAc, 50:50:7 and, after destaining in the same solvent, stored in 7% acetic acid. Gels were quantitatively scanned with a Joyce–Loebl microdensitometer. Using a red filter, 4 scans were made of each gel turning it through 90° between each scan. Recordings were made on heavy paper with the ratio arm at 2× magnification. The proportion of protein in each band was estimated by cutting out and weighing the peaks.

Brief trypsin treatment of PVX and PVX-Q. To virus (0.3–1.0 g/l) suspended in ammonium bicarbonate (0.1 M) was added 1/100 th of its weight of trypsin (1 g/l. in 1 mM HCl) and the mixture incubated at 20°. At various times, portions of the reaction mixture were mixed with an equal volume of 0.1 M sodium phosphate pH 7.1 containing 2% w/v SDS, 2% v/v 2-mercaptoethanol and 8 M urea, in order to stop the reaction and to prepare protein–SDS complexes for gel analysis. The solutions were usually concentrated by freeze-drying to produce small volumes for analysis by gel electrophoresis.

Exhaustive trypsin-digestion of PVX-Q. PVX-Q was suspended in ammonium bicarbonate (1% w/v; pH 8.5; 0.2–0.34 ml/10 mg protein), and trypsin (DCC-treated; Sigma Chemicals), suspended in ammonium bicarbonate, added in three lots in the course of a 24 hr incubation at 37°. The final solution (0.5–0.9 ml; ratio trypsin: PVX-Q 1:25–30) was freeze-dried, resuspended in water (0.2–0.3 ml), centrifuged to remove coloured, insoluble material, and spotted onto Whatman 3 MM paper. Electrophoresis was performed in a Shandon High Voltage apparatus, either at pH 6.5 (pyridine–HOAc–H_2O, 25:1:225) or at pH 1.9 (HCO_2H–HOAc–H_2O; 1:4:45). It was continued for approx 1 hr at 4 kV. The papers were subsequently dried and cut into strips for the determination of free or total quinic acid or for staining with ninhydrin (0.1% w/v in Me_2CO). The same procedure was used when digests were chromatographed in BuOH–pyridine–HOAc–H_2O (30:20:6:24).

Quinic acid estimations. Quinic acid was estimated by reaction with periodate and thiobarbiturate following the procedure of Mrs. V. K. Newby (unpublished) which is essentially that of Levy and Zucker [21]. It proved unnecessary to extract the pink colour into iso-amyl alcohol before measuring its $E_{549\ nm}$. Esterified quinic was released by saponification before it was estimated [22]: following the procedure of Mr. L. S. C. Wooltorton (unpublished), CA (0.05–0.275 mg) or PVX-Q (5–12 mg) was saponified overnight in 1 ml N—NaOH in the cold (5°) room. The reaction was usually performed in an evacuated Thunberg tube, although, with the samples used, this was not necessary. The solution was neutralized with 1 ml N—H_2SO_4 while still under vacuum. Any protein precipitated was removed by centrifugation and a suitable sample of the supernatant, usually 0.25 ml, taken for the colorimetric assay. These procedures were also used satisfactorily to detect quinic acid and quinic-containing peptides on strips (0.5 × 3.5 cm) of 3 MM paper following electrophoresis. For free quinic acid determinations, the paper was removed from the reaction mixture after the addition of thiobarbituric acid. For bound quinic acid determinations, saponification was done aerobically, partly for convenience and partly because it produced less background colour from the paper, and the paper removed by centrifugation before a sample

(1 ml) was taken for the assay. Small amounts of colour were measured on a Cecil CE 272 spectrophotometer.

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