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CHANGES IN BULK PROTEIN OF TOBACCO LEAVES ON AEROBIC AUTOLYSIS: HYDROGENATION STUDIES AND IDENTIFICATION OF BOUND QUINIC ACID

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; hydrogenation; protein; amino acids; aluminium; chlorogenic acid; quinic acid; rhodium.

Abstract—During aerobic autolysis and in commercial curing, the bulk proteins of tobacco leaves become coupled with quinic acid, presumably in consequence of coupling of chlorogenic acid congeners with lysine ε-NH_2 groups. Quinic acid derivatives, prepared from acid hydrolysates of such altered proteins, were identified by GC–MS. Such proteins were also hydrogenated over Rh/Al_2O_3 with a view to stabilizing the hypothetical linkages. Difficulties in removing contaminant Al had to be overcome. Evidence was then obtained (by GLC of derivatives) for several components, in acid hydrolysates of hydrogenated altered proteins, which were neither normal hydrogenation products of the common amino acids nor derivatives of quinic acid. Details of the chromatograms and mass spectra of quinic acid derivatives are provided in a supplementary publication‡.

INTRODUCTION

There is now a considerable accumulation of evidence that nutritionally important amino acid residues in proteins may undergo deleterious modification in the presence of oxidizing polyphenols [1–4]. In particular, such reactions may diminish the availability of lysine in the products of 'green-crop fractionation' [5]. In an earlier paper from this laboratory [6], model experiments were described which suggested that catalytic hydrogenation may prove a useful technique for chemically stabilizing, and thus identifying, the alteration products from lysine residues. A more recent paper [3] gave evidence, mainly based on a colour reaction, for the coupling of chlorogenic acid congeners with leaf proteins; there was simultaneous diminution of their 'total' and 'chemically available' lysine contents. These effects were particularly strong in tobacco leaves (*Nicotiana tabacum* L.) undergoing aerobic autolysis. In the present paper we describe the techniques which we have developed for isolating some of the various hydrolysis products from such hydrogenated proteins, and for assessing the extent of hydrogenation of the normal protein constituents. We have also, by GC–MS of derivatives, obtained more direct evidence that quinic acid (presumably as a moiety of chlorogenic acid congeners) becomes bound to tobacco-leaf proteins during aerobic autolysis.

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‡ SUP 90038. *Annex* 1. Gas–liquid chromatograms of derivatized materials (J.F.M.); *Annex* 2. GC–MS identification of quinic acid derivatives (J. Eagles and J.F.M.) 24 pp. in all. Deposited at the British Lending Library, Boston Spa, Yorkshire, LS23 7BQ, from whom copies can be obtained. Supplementary Publication Scheme—Information which supplements this article has been deposited with the National Lending Library, Boston Spa, Yorkshire, LS23 7BQ, U.K. This supplementary information is available as microfiche or as enlargements from the Library's photocopying services.

RESULTS AND DISCUSSION

Purification of amino acids and their derivatives after hydrogenation of proteins

The hydrogenation conditions which had been found satisfactory [6] involved the use of rhodium metal (deposited on aluminium oxide) suspended in 70% (v/v) aqueous acetic acid, which has proved a good solvent for bulk plant proteins [6, 7]. In the course of reaction, substantial dissolution of the aluminium oxide occurred. Dissolved Al^{3+} was well retained on cation-exchange resin columns, and did not interfere with amino acid analyses of acid hydrolysates under Moore and Stein's conditions. However, some of the substances for which we were searching could not be expected to leave the column under these conditions. We did not explore elution with buffers of higher pH. However, attempted desalting by absorbing the amino acids of a hydrolysate onto H^+-resin and subsequently eluting them with aqueous ammonia led to substantial simultaneous elution of aluminium, and therefore was not pursued further. An attempt to desalt the hydrogenated protein *before* hydrolysis, by chromatography on G-25 Sephadex in 70% (v/v) aqueous acetic acid, gave substantial, but still incomplete removal of aluminium. This (presumably by 'mordanting' effects) interfered with zone-electrophoretic separations on filter paper, when these were attempted on the protein hydrolysate. An attempt to remove Al cations by electrodialysis of the hydrogenated protein in a 3-compartment cell (with cellophan membranes) in 70% acetic acid was likewise unsuccessful. Perhaps these troubles could have been avoided by using rhodium deposited on carbon for the hydrogenations.

We eventually got satisfactory results by hydrolysing

the protein in the presence of the aluminium salt and then converting the amino acids of the hydrolysate to their heptafluorobutyrylated *n*-propyl esters [8]. These were then dissolved in chloroform for successive washing with weakly alkaline and acidic aqueous phases, which removed the aluminium compounds. The derivatives remaining in the chloroform phase could then be studied by GLC [8]. Such derivatives show, on electron-impact MS, substantially more complex fragmentation patterns than those given by acetylated amino acid methyl esters [9, 10]; this hinders deduction of structures, at least until more experience with MS of this group has been gained. We accordingly tried to free acetylated methyl esters of amino acids from aluminium by the above procedure. Substantial losses of these derivatives into the aqueous phases were incurred. Later, by model experiments, we found that free amino acids can be regenerated in good yield by acid hydrolysis of their heptafluorobutyrylated *n*-propyl esters, making them available for rederivatization with whatever groups seem most suitable [10].

Derivatization and GLC of acid hydrolysates of bulk leaf-protein preparations gave several zones not attributable to amino acids. We took these to originate from fatty [11] and aromatic [12] acids bound to the leaf proteins and, occasionally, from plasticizers present as contaminants of our apparatus, reagents or solvents. With acid hydrolysates of fresh tobacco-leaf bulk protein, carefully prepared under nitrogen [7], extraction of the hydrolysate with chloroform removed all these zones, and only zones attributable to the common amino acids were seen [8]. Such initial chloroform extraction was therefore always done routinely, before derivatization.

Identification of quinic acid derivatives

When 'aerobically autolysed' tobacco-leaf bulk protein or cigar-tobacco protein was worked up, several new GLC zones were encountered (Fig. 1c). In view of previous evidence for binding of chlorogenic acid to the protein [3], a model experiment was done in which quinic acid was subjected: (a) to derivatization, washing and GLC (Annex 1): (b) to acid hydrolysis, as for protein, followed by the entire routine analytical procedure (Fig. 1a). Co-chromatography (Fig. 1b) of this last mixture with the mixture of Fig. 1c showed that *all* the unknown zones therein could be explained as derived from quinic acid. Presumably these various zones are derivatives of products of epimerization [13, 14], lactonization (quinides) [13, 14] and/or dehydration (shikimic acid and its epimers) [15]. The novel zones (Fig. 1c) from cigar-tobacco protein gave MS closely similar to those from the letter-marked zones (Fig. 1a) derived from quinic acid (Annex 2). Experiments by partition chromatography on cellulose [16] designed to detect hydroquinone and *p*-hydroxybenzoic acid (reported on heating quinic acid in HCl at much higher temperatures [17]) gave negative results. It was further shown, by their non-retention on a cation-exchange resin, that the substances yielding the GLC zones supposed to be derived from quinic acid (Fig. 1) were devoid of basic groups (see Experimental). These were the only GLC zones found after the ion-exchange treatment. It thus seems that quinic acid congeners are the only water-soluble, chloroform-insoluble acidic or neutral compounds which are released from the aerobically-autolysed protein fractions on acid hydrolysis and give, on derivatization, GLC zones having retentions in the range under study.

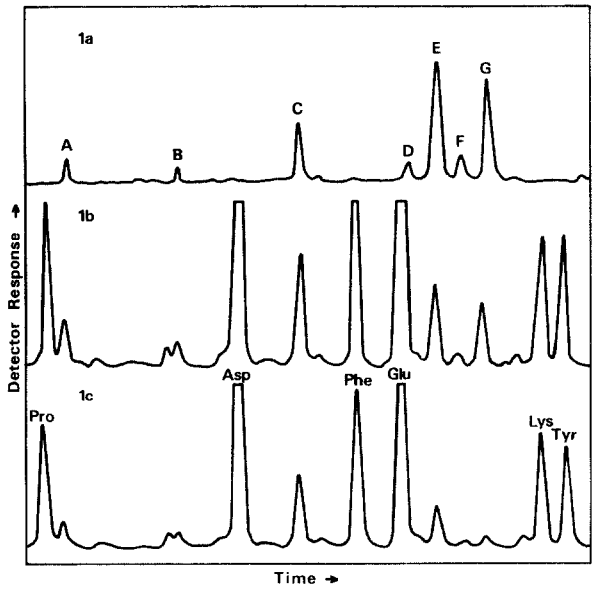

Fig. 1. Segments of gas–liquid chromatogram profiles relevant to quinic acid from derivatized materials [8]: (a) quinic acid subjected to protein-hydrolysis treatment; (b) co-chromatogram of (a) with (c); (c) hydrolysate of cigar-tobacco protein. Zones A–G are discussed in text. For more complete treatment, see Supplementary Publication.

Extent of hydrogenation of the 'protein' amino acids

The GLC technique, as thus developed, gave a good measure of the conversion of methionine to 2-aminobutyric acid (Annex 1). However, the main hydrogenation product of phenylalanine and tyrosine, 3-cyclohexylalanine, gave a zone coinciding in position with that from phenylalanine [8]. Minor hydrogenation products from tyrosine, possibly hydroxycyclohexylalanines, were suggested by GLC; model experiments showed other new zones to coincide with diastereoisomers of octahydrotryptophan (Annex 1). The extent of conversion of phenylalanine and tyrosine to 3-cyclohexylalanine derivatives was therefore better ascertained by ion-exchange chromatography, 60–85% conversion being usually encountered [6]. These were the only changes noted on hydrogenation of 'fresh' tobacco-leaf bulk protein (Annex 1).

Hydrogenation products from 'aerobically autolysed' tobacco-leaf bulk protein

After hydrogenation of 'aerobically autolysed' protein, several 'new' zones were found in addition to those already shown to be derived from quinic acid and discussed above. Zones in the same positions were found, more prominently, with preparations from cigar. These are being studied in more detail, in the hope that some of them may prove to be derived from reaction products of the ε-NH_2 group of lysine residues. For this purpose, it is proposed to conduct the GLC on a preparative scale, with trapping [18] of zones of interest. Trapped fractions can be hydrolysed to their parent amino acids, whose derivatives may then be studied by MS after methyl esterification, acetylations, permethylation, with deuteriosubstitution as desired [10]. There was no sign, on ion-exchange chromatograms of hydrolysates of cigar protein (see Experimental), suggesting the presence

of homocitrulline or 2-aminoadipic acid, reported from tobacco protein by Weybrew and Long [19] and stated by them to be 'metabolites of lysine'. Perhaps there had been foliar application of urea to the tobacco studied by them, and not to our Cuban tobacco. Neither did we find either amino acid by GLC ([8] and Annex 1) in the protein from the aerobically-autolysed tobacco leaves grown at this Institute.

EXPERIMENTAL

Materials. Tobacco-leaf protein ('fresh' and 'after aerobic autolysis') and cigar protein were the 'Bag contents' fractions described in ref. [3]. The hydrogenation catalyst was 'rhodium 5% on alumina; catalyst grade' (Koch-Light) [6]. DL-Homocitrulline was prepared according to Kurtz [20]. Other chemicals were obtained commercially.

Hydrogenation and hydrolysis. These were done as described for 'bulk protein of potato tuber' [6]. Five or six additions of catalyst, on successive days, were usually required before hydrogen uptake diminished. Acid hydrolysis was usually done under reflux, not in a sealed tube.

Removal of lipids etc., derivatization, removal of Al *and GLC.* Acid hydrolysates of protein fractions etc. (whether hydrogenated or not) were evapd to dryness *in vacuo* with repeated additions of H_2O until HOAc and HCl were largely removed. To the residue was added 0.5 M HCl (5 ml) and the resulting mixture was extracted ×3 with redistilled $CHCl_3$ (B.P., 5 ml portions). The $CHCl_3$ extracts were discarded and the aq. phase was made to vol. (10 ml) with water. Suitable aliquots of this soln were subjected to derivatization [8] with the modifications that: (i) for hydrogenated materials containing Al salt, the treatment with propanolic–HCl was repeated once; (ii) 2,6-di-*t*-butyl-*p*-cresol (BHT) was omitted at the acylation stage, since it gave its own substantial zone in an interesting region of the gas chromatograms and since we were not concerned with quantitative study of methionine and histidine. The oily residues, after evapn of the acylation reagent with dry N_2, were taken up in $CHCl_3$ (10 ml) and the $CHCl_3$ phase was extracted successively with 0.1 M $NaHCO_3$ (5 ml), 0.1 M HCl (5 ml) and H_2O (10 ml). These aq. extracts were discarded and the washed $CHCl_3$ phase was evapd to dryness at first *in vacuo* (with the flask in an ice–H_2O bath) and finally in a desiccator over H_2SO_4/NaOH at room temp. and atmos. pres. The resulting residue was available for GLC [8] experiments.

Hydrolysis of derivatized amino acids: estimation of recovery by ion-exchange chromatography. An acid hydrolysate of cigar protein (*ca* 1 mg), which had been derivatized as above, was then subjected to hydrolysis by refluxing for 24 hr in a mixture of 10 M HCl (2.4 ml) and HOAc (1.6 ml). The hydrolysate was evapd to dryness *in vacuo* with repeated additions of H_2O and an aliquot of the residue was analysed for amino acids on a Beckman 120C Amino Acid Analyzer [21]. Recovery of cystine was 14%, of the other amino acids in the range 47 (alanine) to 94% (lysine), based on the GLC [8] figures for the derivatized mixture taken.

Ion-exchange stripping of amino acids from protein hydrolysate. An acid hydrolysate of hydrogenated cigar protein (*ca* 2 mg) was evapd to dryness, the residue was taken up in H_2O (1 ml) and filtered through a 1 ml bed of H^+-sulphonated-polystyrene resin (AG 50W–X8, 200–400 mesh/in., Bio-Rad Labroatories, Richmond, Calif., U.S.A.). The bed was then washed with H_2O (5 ml). The total aq. extract, on evapn and derivatization as above, gave a GLC profile showing all the zones (and only such zones) at the positions seen in Fig. 1a.

Detection of 2-aminoadipic acid and homocitrulline by ion-exchange chromatography. Mr. A. M. C. Davies writes: "Separation of these amino acids was achieved on a Technicon TSM amino acid analyser equipped with a recently developed microcomputer-controlled gradient-elution device [22]. Gradients of pH and sodium molarity were used to separate 2-aminoadipic acid ahead of glycine and to elute homocitrulline between cystine and valine. Neither amino acid was detected in the cigar-protein hydrolysate but addition of the amino acids to the sample produced peaks in the appropriate positions".

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