Isolation of 6-Hydroxykynurenic Acid from the Tobacco Leaf

By P. K. MACNICOL

Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization,
Canberra, A.C.T., Australia

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1. 6-Hydroxykynurenic acid (4,6-dihydroxyquinoline-2-carboxylic acid, 6-HKA) was isolated in crystalline form from both green and cured tobacco leaves. 2. A method for the determination of 6-HKA by paper chromatography and fluorimetry is described. 3. The content of 6-HKA in the flowers, stem and roots of the tobacco plant was much lower than that in the leaf. 4. The 6-HKA content increased throughout leaf development and senescence. 5. 6-HKA was detected in the leaves of plants representing 11 out of 27 families sampled. 6. 6-HKA was found to be devoid of antibacterial and antifungal activity, and was inactive in the *Avena*-coleoptile and cress-seed-germination tests. 7. The presence of 6-HKA is taken as evidence in plants of the tryptophan-catabolic pathway already known in mammals and micro-organisms.

During a chemotaxonomic study of phenolic constituents of the genus *Nicotiana* attention was attracted to a compound, on paper chromatograms of leaf extracts, showing a strong pink fluorescence in ultraviolet light. Although the presence of this constituent was noted by previous investigators (Reid, 1956; Weaving, 1958), its structure was not clarified.

The 'quality' of the cured tobacco leaf is a subjective evaluation based on criteria such as colour and aroma, and is intimately related through its chemical composition to the pre- and post-harvest biochemistry of the leaf. It has long been known that 'quality' is positively correlated with total phenolic content (Bruckner, 1936). More recently some basis for this has been given by the finding (Wright, Burton & Berry, 1962) that cured tobacco leaf contains pigments of high molecular weight that are complexes of oxidized phenols, iron and protein. As a contribution to the chemical characterization of leaf 'quality', the isolation of the pink-fluorescing phenol was undertaken. It was early found not to be identical with cichoriin (aesculetin 6- β -glucoside), which exhibits similar fluorescence and was detected in tobacco by Runeckles (1962). The new compound has been identified as the simple quinoline derivative 6-HKA,* and its widespread occurrence raises questions about the catabolism of tryptophan in plants.

* Abbreviations: 6-HKA, 6-hydroxykynurenic acid; p.m.r., proton magnetic resonance.

MATERIALS AND METHODS

Melting points were determined in a Büchi apparatus and are corrected. C, H and N ultramicroanalyses were kindly performed by Dr W. Walisch, Universität des Saarlandes, Saarbrücken, Germany. Ultraviolet-absorption spectra were measured with an Optica CF4 spectrophotometer and infrared-absorption spectra with a Beckman IR7 (for KBr micro-pellets) or a Perkin-Elmer 337 spectrophotometer (solutions). The p.m.r. spectrum of the natural product in [D₆]dimethyl sulphoxide was measured in a Varian HR-100 spectrometer, and that of its methyl derivative in CCl₄ in a Varian A-60 spectrometer. The chemical shifts are expressed relative to the internal standard tetramethylsilane ($\delta=0$). The mass spectrum was determined with an MS9 spectrometer at 70 eV. The fluorescence spectrum and quantum efficiency of fluorescence (relative method) were measured with a spectrofluorimeter designed and constructed in the C.S.I.R.O. Division of Plant Industry, Canberra, A.C.T., Australia.

Plant material. Field-grown tobacco plants (*Nicotiana tabacum* L.) were of the cultivar Hicks and were grown in North Queensland under the prevailing conditions of commercial practice. Glasshouse-grown tobacco plants of the variety GA955 (resistant to *Peronospora tabacina* Adam) were raised at 35° in peatmoss-sand mixture, with nutrient tablets. Leaf material of other plant species was obtained from plants growing wild or in gardens.

Paper chromatography. The following solvent systems were used for ascending chromatography: (a) ethyl acetate-pyridine-water (12:5:4, by vol.); (b) butan-1-ol-pyridine-water (14:3:3, by vol.); (c) propan-2-ol-formic acid-water (5:1:94, by vol.); (d) propan-2-ol-aq. NH₃ (sp. gr. 0.88)-water (10:1:1, by vol.); (e) butan-1-ol-acetic acid-water (4:1:5, by vol.; organic phase); (f) 10% (v/v) acetic acid;

(g) phenol saturated with water. 6-HKA was detected by its pink fluorescence in short-wave or long-wave u.v. light.

Thin-layer chromatography. Layers (usually 250 μ in thickness) of silica gel G (E. Merck A.-G., Darmstadt, Germany), activated overnight at 90°, were used in conjunction with the following solvent systems: (b) above; (h) chloroform-methanol (9:1, v/v); (i) chloroform-acetic acid-water (4:4:1, by vol.).

Synthesis of 6-HKA. The method of Makino & Takahashi (1954), which utilizes *p*-anisidine and diethyl oxaloacetate as starting materials, was employed with minor modifications. The hydroiodide was converted into the free base by precipitation with *N*-HCl (to approx. pH 2) from 0.5M- Na_2CO_3 solution. The precipitated 6-HKA was washed with ice-cold water until chloride could no longer be detected in the supernatant, and was dried over P_2O_5 . The pale-tan powder had m.p. 283° (decomp.) (Found: C, 57.8; H, 3.55; N, 6.7. Calc. for $\text{C}_{10}\text{H}_7\text{NO}_4$: C, 58.5; H, 3.42; N, 6.8%).

Determination of 6-HKA in leaf material. Freshly punched-out leaf disks (1g.) were dropped into 5ml. of boiling 80% (v/v) ethanol in a test tube fitted with a cold-'finger' condenser, and boiling was continued for 3min. After cooling, the disks and supernatant were ground in a Potter homogenizer and the homogenate was filtered under suction with the aid of Celite. The residue was transferred to the original tube and refluxed for 3min. in 5ml. of 70% (v/v) ethanol. The suspension was filtered hot and washed with small volumes of hot 70% ethanol until the filtrate was colourless. The combined filtrate was extracted with an equal volume of light petroleum (b.p. 60–80°), and the aqueous phase was concentrated almost to dryness in a rotary film evaporator at 40° and made up to exactly 1ml. with 50% (v/v) ethanol. A 50 μ l. sample was then spotted on to Whatman no. 3 paper and subjected to two-dimensional chromatography in solvent systems (b) (first direction) and (c) (second direction). The 6-HKA spot was located under u.v. light and cut out, as was an adjacent blank piece of approximately the same size. The pieces were weighed, cut into small squares and eluted with 5ml. of 50% (v/v) methanol in stoppered tubes that were mechanically shaken for 2hr. at 50°. After cooling, the eluates were decanted and their fluorescence was determined in an Eppendorf photometer with fluorescence attachment, 366 $m\mu$ primary filter, 420–3000 $m\mu$ secondary filter and 1 cm. quartz cells. The fluorescence of the test sample was corrected for the solvent- and paper-blank fluorescence, and the 6-HKA content was obtained from a calibration curve made with synthetic 6-HKA in 50% methanol. This was linear in the range 0.002–0.1 $\mu\text{g.}/\text{ml.}$ The recovery of known amounts (2–5 $\mu\text{g.}$) of 6-HKA added to the leaf material and put through the whole procedure was 85–95%.

Chlorophyll determination. Total chlorophyll was determined by the method of Bruinsma (1961).

Isolation and properties of 6-HKA from tobacco leaves. Technically ripe to over-ripe leaves from field-grown plants (cultivar Hicks) were oven-dried at 70–80° and the midribs removed. The laminae (12kg.) were crushed to a coarse powder and extracted for 1hr. with 85l. of boiling 80% ethanol. To the cooled filtered extract were added 0.5 vol. of chloroform and 0.5 vol. of water. After thorough stirring and separation of the phases, the lower phase was discarded. The upper phase was treated with 1.0kg. of activated

charcoal powder (British Drug Houses Ltd., Poole, Dorset), and after the suspension had been stirred for 2hr. the charcoal was filtered off and washed with 5l. of 50% ethanol. Elution of the adsorbed 6-HKA and other compounds was carried out by suspending the charcoal in 3l. of ethanol-pyridine-water (1:1:1, by vol.), heating at 70° for 10min. and filtering. This procedure was repeated three times. The combined eluate was freed from pyridine and ethanol in a rotary film evaporator at 50° by repeated addition of water and finally concentrated to 300ml. After adjustment of its pH to 2.5 with 5N- H_2SO_4 , the concentrate was extracted six times with 1 vol. of pentan-1-ol. The extract was concentrated tenfold on the rotary film evaporator and subjected to partition chromatography on 30cm. $\times$ 3.5cm. columns of cellulose powder (Whatman) in the system light petroleum (b.p. 100–120°)-ethyl acetate-acetic acid-water (15:20:4:1, by vol.). The effluent was collected in 200ml. fractions and those containing the 6-HKA (peak effluent volume approx. 2.2l.) were combined. The 6-HKA, accompanied by scopolin and chlorogenic acid [detected by paper chromatography in solvent (a)], was recovered from the combined fractions by shaking them with 0.1 vol. of water. The organic phase was discarded, the aqueous phase evaporated to dryness and the residue dissolved in 50ml. of water. After adjustment of its pH to 2.5, the solution was extracted six times with pentan-1-ol as before. The combined extract was concentrated to 30ml. and chromatographed on a 30cm. $\times$ 3.5cm. cellulose column with the system light petroleum (b.p. 100–120°)-ethyl acetate-pyridine-water (5:10:5:1, by vol.). The elution of 6-HKA could be followed under the u.v. lamp by virtue of its yellow fluorescence in this solvent; the peak effluent volume was approx. 6l. Although paper chromatography in solvent (a) indicated that the eluate was now free from other fluorescent compounds, attempts to crystallize the 6-HKA failed. The final purification step consisted of anion-exchange chromatography on DEAE-cellulose. The cellulose-column eluate was shaken with 0.05 vol. of water and the aqueous phase evaporated to dryness, with repeated addition of water to remove the pyridine. The residue was suspended in 30ml. of 0.1N-acetic acid, filtered, and passed through a 10cm. $\times$ 1.0cm. column of DEAE-cellulose (Cellex D; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) in the acetate form, equilibrated with 0.1N-acetic acid. For elution 2N-acetic acid was employed; the 6-HKA was seen in u.v. light to move slowly down the column as a broad yellow band. Crystallization resulted when the eluate was left overnight in the refrigerator. Recrystallization from 2N-acetic acid yielded 10mg. of clusters of minute yellowish white needles, which were dried under vacuum over NaOH pellets. A second isolation, from 31kg. of cured field-grown leaves, yielded 28mg. of crystalline material with identical physicochemical characteristics.

The compound had m.p. 287° (decomp.) (Found: C, 57.7; H, 3.47; N, 6.7. Calc. for $\text{C}_{10}\text{H}_7\text{NO}_4$: C, 58.5; H, 3.42; N, 6.8%). It was insoluble in benzene, chloroform and ether, sparingly soluble in water and methanol, and soluble in pyridine and dimethylsulphoxide. In solution it exhibited a characteristic bluish-pink fluorescence in u.v. light (emission maximum in methanol at 440 $m\mu$ with excitation at 350 $m\mu$; quantum efficiency approx. 0.17), changing to yellow when it was ionized. On paper the fluorescence was light pink and amounts as low as 0.01 $\mu\text{g.}$ could be detected visually. Chromatographic R_F values in various solvent systems are

given in Table 1. The compound was stable to boiling for 2 hr. in aqueous 2N-HCl and to standing for 2 hr. at room temperature in methanolic 2N-NaOH. Details of its u.v. and mass spectra are given in Tables 2 and 3 respectively, and its i.r. spectrum is shown in Fig. 1. The p.m.r. spectrum is discussed below.

Methyl derivative. A methanolic solution of the natural product (2mg.) was treated with excess of ethereal diazomethane at room temperature. Unchanged 6-HKA, detected by paper chromatography in solvent (a), was still present after 3 hr. but not after standing overnight. The reaction mixture was evaporated to a dryness in a rotary film evaporator and the residue extracted with chloroform. The extract was concentrated to a small volume and streaked on to a 20 cm. x 20 cm. plate bearing a 1 mm. layer of silica gel. Development in solvent (h) allowed separation of the presumed fully methylated derivative, showing a blue fluorescence (R_F approx. 0.9), from three other major

products fluorescing yellow-green, blue and pink (R_F values approx. 0.8, 0.7 and 0.5 respectively) and several minor fluorescent ones. On recovery from the silica gel, the fastest-moving derivative crystallized from *n*-hexane as colourless prisms. The small quantity obtained precluded elemental analysis. The p.m.r. spectrum in CCl_4 showed peaks at $\delta=3.88$, 3.92 and 4.08 p.p.m., confirming the presence of three methoxyl groups. The main absorption bands in the i.r. spectrum in CCl_4 were at 2930, 1720, 1480, 1360, 1280,

Table 3. *Mass spectrum of 6-HKA isolated from tobacco*

Peak (m/e)	Rel. intensity (base peak=100)	Metastable peak	Probable transition
205	42		
187	3		
		170.6	205 $\xrightarrow{-H_2O}$ 187
161	100		
159	35		
145	*		
144	2		
		135.2	187 $\xrightarrow{-CO}$ 159
133	21		
		127.0	205 $\xrightarrow{-CO_2}$ 161
		125.7	
121	25		
120	*		
		110.0	{ 161 $\longrightarrow$ 133
		108.0	{ 133 $\longrightarrow$ 121
105	22		
104	17		
		103.3	
		90.8	
		83.0	
44	25		

* A small peak appeared with time.

Table 1. *R_F values of 6-HKA on paper chromatograms and on thin-layer chromatograms*

Whatman no. 1 paper and silica gel G were used. For details of solvents see the Materials and Methods section.

Solvent	Paper chromatography		Thin-layer chromatography	
	Natural compound	Synthetic compound	Natural compound	Synthetic compound
(a)	0.31	0.30		
(b)	0.14	0.13	0.53	0.53
(c)	0.39	0.39		
(d)	0.05	0.06		
(e)	0.53	0.53		
(f)	0.51	0.51		
(g)	0.49	0.52		
(h)			0.00	0.00
(i)			0.63	0.62

Table 2. *Ultraviolet-absorption maxima of 6-HKA and its fully methylated derivative*

The maxima given for 6-HKA apply to both the synthetic and the natural compound; extinction coefficients were determined only for the latter. sh., Shoulder.

	λ_{max} (m μ)		
	Methanol	0.1 N-NaOH in methanol	0.1 N-HCl in methanol
6-HKA	213 (log $\epsilon=4.26$)	211 (log $\epsilon=4.76$)	213 (log $\epsilon=4.29$)
			226 (sh.)
	256 (log $\epsilon=4.37$)	256 (sh.)	260 (log $\epsilon=4.46$)
	347 (log $\epsilon=3.86$)	263 (log $\epsilon=4.34$)	
	362 (sh.)	385 (log $\epsilon=3.86$)	363 (log $\epsilon=3.81$)
Fully methylated derivative	212	213	213
			227 (sh.)
	253	252	261
	316	316	
	330 (sh.)	330 (sh.)	326 (sh.)
			358

Fig. 1. Infrared-absorption spectra (KBr micro-pellet) of 6-HKA from tobacco leaves (upper trace) and synthetic 6-HKA (lower trace).

1240 and 1110 cm.⁻¹. The u.v.-absorption maxima are summarized in Table 2.

Taxonomic distribution. Care was taken to include leaves of a wide range of physiological age in each sample. The material was extracted with hot 70% ethanol and chloroplast pigments were removed from this extract by shaking with chloroform. The resulting aqueous phase was concentrated in a rotary film evaporator and a sample equivalent to 50 mg. fresh wt. was chromatographed two-dimensionally in solvent systems (b) and (c). 6-HKA, where present, was detected by its characteristic pink fluorescence in u.v. light and its R_F values (see Table 1); the lowest limit of detection by this method corresponded to a 6-HKA content in the leaf of approx. 0.2 μg./g. fresh wt.

Tests for biological activity. Antibacterial activity (200 p.p.m. of 6-HKA in Difco heart infusion broth) was tested against *Staphylococcus aureus* N.C.T.C. 4163, *Pseudomonas aeruginosa* N.C.T.C. 6749, *Escherichia coli* N.C.T.C. 8196, *Salmonella typhi* N.C.T.C. 786 and *Klebsiella pneumoniae* type 3. Antifungal activity was tested against *Peronospora tabacina* Adam (spore germination; 100 p.p.m. of 6-HKA in agar), *Monilinia fructicola* (Wint) Honey (spore germination; 50 p.p.m. in solution containing glucose and inorganic salts), *Aspergillus nidulans* (Eidam) Wint (spore germination; 50 p.p.m. in solution containing glucose, glutamic acid and inorganic salts) and *Phytophthora palmivora* Butl. (growth; 30 p.p.m. in potato-dextrose agar). The *Avena*-coleoptile test was carried out by the method of Kefford & Caso (1966), with Svalof Victory oats and 0.8 mm-6-HKA. The cross-seed (*Lepidium sativum* L.) germination test was performed in the dark at 25° in Petri dishes, the seed being placed on filter paper moistened with 1.5 mm-6-HKA.

RESULTS

Structural elucidation. Some preliminary information was obtained by the use of chromogenic sprays on paper chromatograms. A phenolic hydroxyl group was indicated by an instant blue colour with the ferric chloride-potassium ferri-cyanide reagent of Barton, Evans & Gardner (1952). A yellow reaction to bromocresol green (Lugg & Overell, 1948) suggested the presence of a carboxyl group, as did movement to the anode on paper

electrophoresis in acetate buffer, pH 5.2 (Pridham, 1959). No reaction with ninhydrin, isatin or Ehrlich reagents was observed. Failure to develop colour in the magnesium-hydrochloric acid reduction test (Shinoda, 1928) suggested that the compound was not a flavonoid. Stability to hydrolysis by 2N-hydrochloric acid at 100° and by emulsin indicated that it was not an *O*-glycoside.

The u.v. spectra of the compound and its methyl derivative were suggestive of a bicyclic aromatic system, and the bathochromic shift with acid made the presence of a basic nitrogen atom probable. Crucial information was provided by the high-resolution mass spectrum, which gave a mass for the parent peak of 205.0379, corresponding fairly certainly to the formula C₁₀H₇NO₄ (Calc. 205.0375). The immediate loss of mass 44 confirmed the presence of a carboxyl group. Bearing in mind the structures of known natural products, these data suggested the most likely candidate to be a dihydroxyquinolinecarboxylic acid. The p.m.r. spectrum (see below) showed that on this assumption the benzenoid ring must contain two protons respectively *ortho* and *para* to a third proton, and the pyridine ring a single proton isolated from the others.

In a search for previous reports of the natural occurrence of compounds of this type it was found that 6-HKA had been isolated in small amount from pig urine by Roy & Price (1959), who described its fluorescence as 'red-white'. 6-HKA was accordingly synthesized and found to show fluorescence characteristics, chromatographic mobility (Table 1) and C, H and N analyses identical with those of the natural product. A slight discrepancy was observed between these values and the theoretical (the carbon figure was low by 0.7–0.8%), which could not be attributed to water of crystallization; the correctness of the molecular formula was, however, confirmed by the mass-spectroscopic evidence. The i.r. spectra (Fig. 1) were virtually superimposable,

thus establishing the identity of the tobacco compound with 6-HKA. The interpretation of the spectrum is complicated by the undoubtedly tautomeric nature of 6-HKA (e.g. the shoulder at 1650cm^{-1} may represent the 4-quinolonecarbonyl group). The bands at 3460 and 3170cm^{-1} are ascribed to hydrogen-bonded $\cdot\text{OH}$ or $:\text{NH}$ groups (or both), and the band at 1740cm^{-1} is ascribed to the carboxyl carbonyl group. The i.r. spectrum of the methyl derivative supports these assignments. The p.m.r. spectrum and its interpretation are as follows. A singlet at $\delta = 6.64\text{p.p.m.}$ (1H) is assigned to the 3-proton, a doublet at 7.38p.p.m. (1H; $J = 2\text{cyc./sec.}$) to the 5-proton, a doublet of doublets near 7.20p.p.m. (1H; $J = 2\text{cyc./sec.}$ and 9cyc./sec.) to the 7-proton, and a doublet at 7.83p.p.m. (1H; $J = 9\text{cyc./sec.}$) to the 8-proton. In the absence of spectra of related compounds, the mass spectrum (Table 3) is difficult to interpret; there is some indication of two simultaneous fragmentation paths. The losses of water, carbon monoxide and carbon dioxide were confirmed by high-resolution mass measurement of the relevant peaks.

The tautomeric nature of 6-HKA emerges clearly from the u.v. spectra (Table 2). The relatively small bathochromic shift on protonation shown by the free acid, in comparison with that of the fully methylated derivative, indicates that in the former compound the 4-quinolone structure (I) must predominate. This conclusion is supported by the close similarity of the spectra after protonation. The large bathochromic shift in alkali presumably

Table 4. *Distribution of 6-HKA within a field-grown tobacco plant*

Part of plant	6-HKA content (%, dry wt.)
Leaf 20* (over-ripe)	0.0012
Leaf 24 (ripe)	0.0010
Leaf 28 (green)	0.0009
Leaf 32 (green)	0.0009
Flowers	< 0.0001
Stem	< 0.0001
Root	< 0.0001

* Leaf numbers counted from base of plant; total number of nodes approx. 43.

results from conjugation extending between the ionized 6-hydroxyl and carboxyl groups.

Distribution within the plant. As Table 4 shows, 6-HKA occurred principally in the leaf of the tobacco plant, only traces being present in the flowers, stem and roots. Within the leaf, 6-HKA was found to be confined to the lamina.

Table 5. *Relation between 6-HKA content and physiological age of leaves at the same stem position (9) of glasshouse-grown tobacco plants*

Degree of leaf expansion (% of final area)	Total chlorophyll (mg./cm. ²)	6-HKA content (%, dry wt.)
3	21.7	0.0010
40	22.0	0.0009
95	26.4	0.0024
100	14.8	0.0034
100	0.50	0.0061

Table 6. *Taxonomic distribution of 6-HKA*

6-HKA present	6-HKA absent
Solanaceae	Solanaceae
<i>Nicotiana tabacum</i>	<i>Hyoscyamus niger</i>
<i>Petunia hybrida</i> cultivar	<i>Cestrum aurantiacum</i>
<i>Atropa physaloides</i>	Boraginaceae
<i>Cyphomandra betacea</i>	<i>Myosotis sylvatica</i> cultivar
<i>Capsicum annuum</i> cultivar	Gramineae
<i>Solantra grandiflora</i>	<i>Triticum aestivum</i>
<i>Datura stramonium</i>	Iridaceae
<i>Nicandra physalodes</i>	<i>Iris</i> cultivar
<i>Isochroma tubulosa</i>	Cannaceae
<i>Streptosolen jamesonii</i>	<i>Canna</i> cultivar
<i>Brunfelsia calycina</i>	Liliaceae
Scrophulariaceae	<i>Lilium tigrinum</i> cultivar
<i>Antirrhinum majus</i>	Rutaceae
Labiatae	<i>Citrus limonia</i>
<i>Salvia farinacea</i>	Rhamnaceae
<i>Rosmarinus officinalis</i>	<i>Pomaderris apetalum</i>
Rubiaceae	Myrtaceae
<i>Gardenia jasminoides</i>	<i>Callistemon lanceolatus</i>
Acanthaceae	Cucurbitaceae
<i>Acanthus mollis</i>	<i>Cucurbita pepo</i> cultivar
Polemoniaceae	Rosaceae
<i>Phlox paniculata</i>	<i>Rosa</i> cultivar
Compositae	Ranunculaceae
<i>Aster novae-angliae</i>	<i>Ranunculus acris</i>
Verbenaceae	Cruciferae
<i>Verbena hybrida</i> cultivar	<i>Hirschfeldia incana</i>
Primulaceae	Vitaceae
<i>Primula</i> cultivar	<i>Vitis vinifera</i> cultivar
Leguminosae	Cistaceae
<i>Phaseolus vulgaris</i>	<i>Cistus purpureus</i>
Malvaceae	Theaceae
<i>Gossypium hirsutum</i>	<i>Camellia japonica</i> cultivar
	Salicaceae
	<i>Populus nigra</i> cultivar

Change in 6-HKA content during leaf development. To avoid confusing ontogenetic changes with a possible gradient of 6-HKA concentration up and down the plant, sampling was confined to a constant leaf position. Table 5 shows that on a dry-weight basis the 6-HKA content increased continuously during development of the leaf, finally reaching six times that in the young leaf. It is noteworthy that the highest content occurred when chlorophyll breakdown was essentially complete, i.e. when the leaf was fully senescent.

Taxonomic distribution. Table 6 shows the result of a small-scale study of the distribution of 6-HKA in the leaves of species belonging to various families representing the main phylogenetic lines of angiosperms, and of the Solanaceae in particular. 6-HKA was found to occur in 11 of the 27 families. It was absent from representatives of all four monocotyledonous families. In the Solanaceae 6-HKA was widely distributed, and in the genus *Nicotiana* it was detected in the leaves of all of more than 20 species examined.

Biological activity. 6-HKA was found to be devoid of antibacterial and antifungal activity and to be inactive in the *Avena*-coleoptile and cress-seed-germination tests.

DISCUSSION

The isolation of the quinoline 6-HKA adds a new type to the already wide spectrum of simple plant phenols. Although there are reports of the isolation of substituted cinchoninic acids (Beattie, 1908; Sahashi, 1926), 6-HKA appears to be the first kynurenic acid derivative isolated from a plant. Its content (0.001–0.006% dry wt.) in the mature tobacco leaf is lower than those of the other prominent phenolic compounds chlorogenic acid, rutin and scopolin (approx. 2.5%, 0.7% and 0.1% respectively; P. K. Macnicol, unpublished work). It is unlikely to be an artifact of extraction (cf. benzoxazolinone in rye seedlings; Virtanen & Hietala, 1959), since the use of other killing methods such as freezing the leaf material in chloroform-methanol-formic acid at -70° followed by thawing (Bielecki, 1964) did not alter the 6-HKA content determined by subsequent chromatography and fluorimetry.

The 6-HKA content was found to increase throughout development and senescence of the leaf. Flue-curing does not appear to alter the content significantly; this is not surprising in view of the marked specificity of tobacco-leaf phenol oxidase towards *o*-diphenols (Clayton, 1959). (Flue-curing consists in holding the harvested leaves at high humidity and approx. 40° until disappearance of the chlorophyll occurs, followed by progressive lowering of humidity and raising of temperature to approx. 80° to halt enzymic activity.) The 6-HKA content

is likewise not markedly affected by growing the tobacco plants under glass, which drastically lowers the chlorogenic acid content owing to the filtering out of short-wave u.v. radiation (Frey-Wyssling & Bähler, 1957).

Apart from being found in the leaves of all the species of *Nicotiana* and most of the other solanaceous genera examined, 6-HKA is present in numerous other angiosperm families. With the exception of *Phaseolus* and *Gossypium*, its distribution appears to be concentrated in the species of genera belonging to the phylogenetically more highly evolved sympetalous families; however, the scale of sampling is as yet too small for this to be certain. This reservation also applies to any comparison with the taxonomic distribution of the quinoline alkaloids.

Up to the present the known biological occurrence of 6-HKA has been restricted to mammalian and avian urine, where it is accompanied by other kynurenic acid derivatives (Roy & Price, 1959; Kido, Noguchi, Tsuji & Matsumura, 1967). In mammals and bacteria kynurenic acid and derived compounds are formed via kynurenine as a result of ring opening between C-2 and C-3 of tryptophan, catalysed by tryptophan oxygenase (Meister, 1965). The biosynthesis of 6-HKA appears to have been studied only in mouse brain homogenates, which form 6-HKA and other products when incubated with 5-hydroxykynurenine (Makino, Joh & Hasegawa, 1962). However, neither tryptophan oxygenase nor any other enzyme in the pathway has been unequivocally demonstrated in a higher plant, although Wiltshire (1953) obtained spectral evidence for the formation of 3-hydroxykynurenine from tryptophan by pea-seedling extracts. Kowanko & Leete (1962) found that young *Cinchona* plants incorporate radioactivity from [methylene- ^{14}C]tryptophan only into C-2 of quinine, and postulated a pathway involving ring opening between the heterocyclic nitrogen atom and C-2 of tryptophan. This evidence nevertheless did not exclude ring opening between C-2 and C-3. Biosynthesis of the quinoline ring from anthranilic acid is known in *Penicillium* (Luckner & Mothes, 1962), *Pseudomonas* (Luckner & Ritter, 1965) and the higher plant *Skimmia* (Matsuo, Yamazaki & Kasida, 1966), but none of the resulting compounds has a 2-carboxyl or related group.

At what stage the hydroxylation step occurs in the synthesis of 6-HKA by mammals and birds is not known. Hayaishi (1955) showed that 5-hydroxytryptophan is not a substrate for tryptophan oxygenase of a *Pseudomonas* strain. This compound, now known to be a free amino acid in plants (Bell & Fellows, 1966), therefore may not be a precursor of 6-HKA. Hydroxylation at the level of kynurenine receives some support from the fact that

Pseudomonas kynurenine transaminase is active on 5-hydroxykynurenine (Hayaishi, 1955). On the other hand, 6-hydroxylation of 4-quinolone in the rabbit was demonstrated by Smith & Williams (1954). Whereas kynurenic acid is completely metabolized by *Pseudomonas fluorescens* (Behrman & Tanaka, 1959), the quinoline ring system appears to suffer little if any breakdown in the mammal (Meister, 1965). The continuous rise in 6-HKA content of the tobacco leaf suggests passive accumulation indicative of similar stability in the plant. Support for this view is found in the inertness of 6-HKA in a number of biological test systems.

Although in mammals and *Neurospora* tryptophan is an obligatory precursor of the pyridine ring (Meister, 1965), in the tobacco plant it is not a precursor of nicotine (Henderson *et al.* 1959). The widespread occurrence of 6-HKA suggests, however, that at least the early steps in the mammalian and bacterial pathway of tryptophan catabolism may occur in plants, and that a search in them for the enzymes of this pathway might be rewarding.

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