in the smallest amount of water and dried again. Finally it was homogenized, dissolved in double distilled water and dialyzed against double distilled water twice overnight. Both the residue in the dialyzing bag and the dialyzate were tested for the UV-absorbance. It appeared that the liberated N-formylkynurenine derivative is dialyzable, suggesting the presence of the tryptophyl residue near the C-end of the collagen molecule. The dialyzate containing the N-formylkynurenine derivative was thereafter made 0,5 M according to $NaHCO_3$ and heated at 100 °C for 4 h. A part of this reaction mixture (0.1 ml) was directly mixed with 50 μl of saturated solution of dimethylamino-naphtalene-5-sulphonylchloride in acetone in order to estimate the N-terminal amino acid residue. The second aliquot (0.2 ml) was used for testing the homogeneity of the resulting peptide by means of paper chromatography (solvent system n-butanol-pyridine-acetic acid-water 30:20:6:24). The main part of the sample was degraded stepwise by EDMAN's technique, modified by GRAY and HARTLEY[6]. The N-terminal amino acid was determined after each degradation step, as described by GRAY and HARTLEY[6], but the identification of resulting dimethylaminonaphtalene sulphonyl derivatives of amino acids has been performed by means of thin-layer chromatography as described by DEYL and ROSMUS[7]. The following amino acid sequence was found: trp-leu-lys-arg[8].

No difference in the sequence of the resulting peptide was found if, instead of acid-soluble collagen, the insoluble collagen was used.

The analysis of the peptide mixture released from the native insoluble collagen by means of trypsin resulted in the detection and isolation of a tryptophane containing peptide[9]. The sequence of this peptide was determined in the same was as described above and the following sequence was found: glu[8]-trp-leu-lys.

Therefore one can conclude that one of the C-terminal sequences in the native collagen is: glu-trp-leu-lys-arg, which is supported by the fact that DEYL et al.[10] found one C-terminal arginine among 6 C-terminal amino acids of the native collagen molecule.

Zusammenfassung. Es wurde die Lage des Tryptophanrestes in der α_3 Tropokollagenkette bestimmt: Das Tryptophan befindet sich auf dem 4. Platz vom C-Ende der Kette, in der Sequenz trp-leu-lys-arg[8]. Der Vergleich mit dem tryptischen Hydrolysat führte zur Verbreiterung der Sequenz um eine weitere Aminosäure: glu-trp-leu-lys-arg[8].

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Laboratory for Gerontology, Czechoslovak Academy of Sciences, Prague-Krc, and Central Research Institute of Food Industry, Prague-Smichov (Czechoslovakia), 6 March 1967.

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[7] Z. DEYL and J. ROSMUS, J. Chromat. *20*, 514 (1965).
[8] trp, tryptophane; leu, leucine; lys, lysine; arg, arginine; glu, glutamic acid.
[9] J. ROSMUS, O. MIKES and Z. DEYL, unpublished observations.
[10] Z. DEYL, J. ROSMUS and S. BUMP, Biochim. biophys. Acta *140*, 515 (1967).

Cholesteryl Esters in Flue-Cured Tobacco

The constituents of tobacco in all its various forms have received extensive chemical study[1], and several sterols, sterol esters, and sterol glycosides have been reported in tobacco – the sterols commonly obtained being stigmasterol, β-sitosterol, ergosterol and campesterol (JOHNSTONE et al.[1,2]). Cholesterol, often considered to be an 'animal' sterol, has been found only recently in plants and to our knowledge has been conclusively identified in only 4 species[3] although its presence has been inferred in others[4]. In spite of extensive work on tobacco, cholesterol and its derivatives have never been isolated from this plant[5]. We now report a simple sequence of separation steps (chart) which results in the ready isolation of a sterol ester fraction from the hexane extract of flue-cured tobacco, together with proof of the presence of a significant percentage of cholesteryl esters in this fraction.

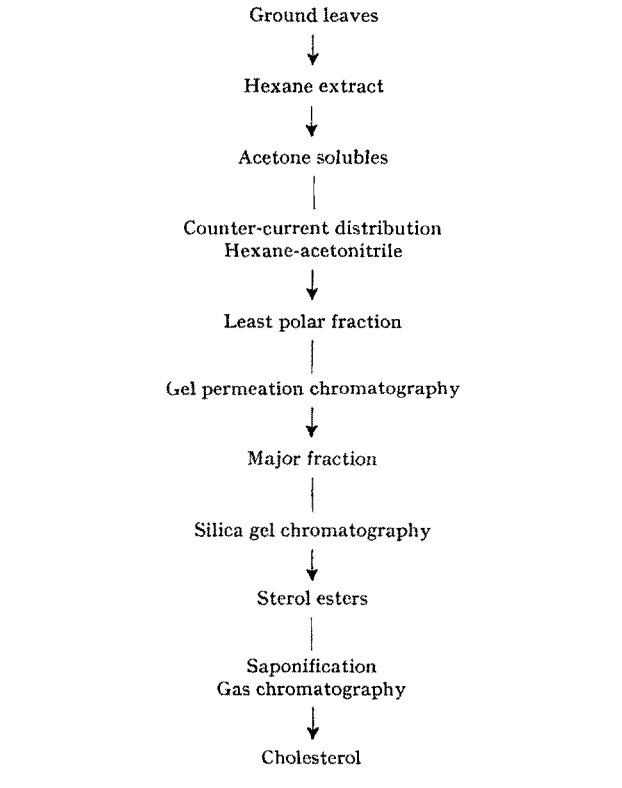
Flue-cured tobacco leaves[6] were ground and extracted continuously with warm hexane[7]. The extract was chilled, filtered, and evaporated, and the residue (5%[8]) was dissolved in acetone and chilled. Precipitated solids were removed by filtration and the acetone evaporated. The residue (4.5%) was subjected to a 14 transfer countercurrent distribution using hexane as the stationary phase and acetonitrile as the mobile phase. The residue from evaporation of tube 0 (i.e., the least polar material, 1.3%)

[1] R. A. W. JOHNSTONE and J. R. PLIMMER, Chem. Rev. *59*, 885 (1959); R. L. STEDMAN, A. P. SWAIN and W. RUSANIWSKYJ, Tob. Sci. *6*, 1 (1962); A. P. SWAIN, W. RUSANIWSKYJ and R. L. STEDMAN, Chem. Ind. 435 (1961).
[2] 'γ-Sitosterol has been shown to be a mixture of β-sitosterol and campesterol. M. J. THOMPSON, W. E. ROBBINS and G. L. BAKER, Steroids *2*, 505 (1963); I. NISHIOKA, N. IKEKAWA, A. YAGI, T. KAWASAKI and T. TSUKAMOTO, Chem. pharm. Bull., Tokyo *13*, 379 (1965).
[3] D. F. JOHNSON, R. D. BENNETT and E. HEFTMANN, Science *140*, 198 (1963); R. D. BENNETT, S. T. KO and E. HEFTMANN, Phytochem. *5*, 231 (1966); B. A. KNIGHTS and W. LAURIE, Phytochem. *6*, 407 (1967); M. DEVYS and M. BARBIER, C. r. hebd. Séanc. Acad. Sci., Paris *261*, 4901 (1965).
[4] See, inter alia, M. F. HÜGEL, W. VETTER, H. ANDIER, M. BARBIER and E. LEDERER, Phytochem. *3*, 7 (1964); P. DUPERON, W. VETTER, M. BARBIER, Phytochem. *3*, 89 (1964); C. DJERASSI, J. C. KNIGHT and H. BROCKMANN JR., Chem. Ber. *97*, 3118 (1964); J. W. ROWE, Phytochem. *4*, 1 (1965).
[5] P. BENVENISTE, L. HIRTH and G. OURISSON reported that the sterol fraction from tobacco tissues grown in vitro contained a minor constituent (1% or less), the molecular weight of which corresponded to that of cholesterol, but conclusive identification was not made. Phytochem. *5*, 31 (1966).
[6] The tobacco was Hicks variety, government grade B4 LV, flue-cured tobacco, harvested and purchased in 1964, and stored in a freezer.
[7] M. Dymicky and R. L. STEDMAN, Tob. Sci. *3*, 179 (1959).
[8] Percentages are approximate and are given in terms of the undried leaf.

was chromatographed on a column of 200–400 mesh polymer beads (polystyrene cross-linked with 2% divinylbenzene[9]) swollen with benzene. Elution was carried out with benzene. Several fractions were obtained, roughly in order of decreasing average molecular weight, as would be expected for this type of chromatography.

The major fraction (0.54%), indicated by IR-spectra to be a mixture of hydrocarbons, esters and alcohols, was dissolved in hexane and adsorbed onto a silica gel column (Davison silica gel, 100–200 mesh, 100:1 weight ratio).

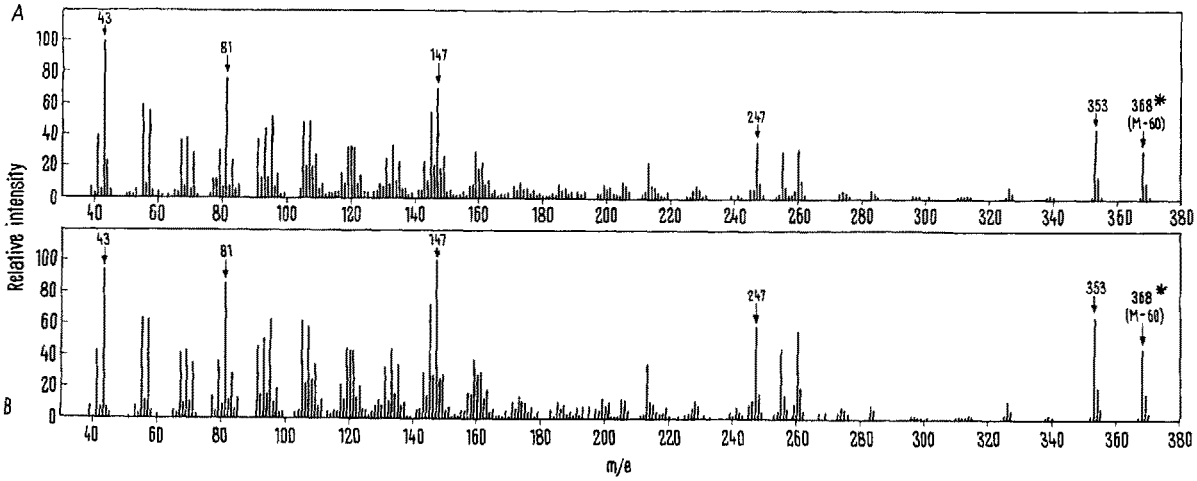
Chart



Elution with hexane removed hydrocarbons. Most of the other material was removed using gradient elution from pure hexane to pure ether. Two main bands were obtained. The more polar of these (0.25%) was shown to be solanesol by its melting point and the melting point and mixed melting point of its *p*-phenylazobenzoyl ester. IR-spectra indicated the less polar fraction (0.11%) to be composed of esters. Separation of this fraction and identification of individual esters was not carried out. Instead the total material was saponified to give an acid fraction (indicated by gas chromatography of the methyl esters to be a mixture of C_{14}-C_{18} fatty acids with palmitic, stearic and oleic acids predominating) and an alcohol fraction. The latter was inseparable from cholesterol and stigmasterol by thin layer chromatography on silica gel in 10% acetone-hexane. Gas chromatography of the alcohols as the dimethylsilyl ethers (column of 1% OV-17 on acid washed, silanized Chromosorb W) showed 4 principal components with relative retention times of 0.74, 1.00, 1.09 and 1.26. These are quite close to the relative retention times reported for the trimethylsilyl ethers of cholesterol, campesterol, stigmasterol and *β*-sitosterol (0.78:1.00:1.10:1.25)[10]. Admixture of genuine cholesterol and stigmasterol followed by silylation enhanced the peaks assigned to these compounds.

The 4 major components were isolated by means of preparative gas chromatography of the dimethylsilyl ethers. The collected substances (each of which gave only 1 peak on gas chromatograms) were boiled with aqueous methanol for removal of the silyl groups and the free alcohols were acetylated. Mass spectra were then obtained. Comparison of the mass spectrum of the acetate from the first peak with that of genuine cholesteryl acetate (Figure) left no doubt as to its identity. The isolation of stigmasteryl, campesteryl and *β*-sitosteryl derivatives is unexceptional, and their identification is based on the gas chromatographic retention times, supported by mass spectra of the isolated acetates which had M-60 peaks at M/e 394, 382 and 396, respectively. (In contrast to the well-separated cholesterol, there was some cross-contamination among these 3 sterols.)

[9] Generously provided by the Dow Chemical Company.
[10] A. ROZANSKI, Anal. Chem. *38*, 36 (1966).



Comparison of mass spectrum of genuine cholesterol with that of sterol isolated from tobacco. (A) Mass spectrum of acetate of GC peak 1, 100° direct probe, 2.0 Kv dynode. (B) Mass spectrum of cholesteryl acetate, 150° direct probe, 2.5 Kv dynode. * Reduced by factor of 10.

The combination of counter-current distribution and gel permeation chromatography[11] provides a convenient and exceptionally mild method for dividing a complex extract into groups of materials which are then amenable to handling by more usual techniques. In this case the sterol esters were separated rather cleanly from other ester material and were contaminated only by materials such as solanesol which were easily separable by conventional adsorption chromatography. Analytical gas chromatography showed that cholesteryl esters represented ca. 5% of the phytosteryl ester mixture. Thus these esters represent about 0.005% of the weight of the tobacco[12].

Zusammenfassung. Nach Hydrolyse wurde Cholesterol von einem Sterolester-Bruchteil aus dem «flue-cured» Tabak isoliert und massenspektrometrisch sein Acetat-Derivat einwandfrei festgestellt, obwohl die einzelnen Ester von Cholesterol im Tabak nicht gereinigt oder identifiziert wurden. Zur Isolierung wurden die Zwei-Stufen-Gegenstromverteilung und die Gel-Permeationschromatographie verwendet, 2 brauchbare und wenig aggressive Methoden zur Aufteilung eines komplexen Extraktes in Materialgruppen und nachfolgender gewöhnlicher chromatographischer Bearbeitung.

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Chemistry and Life Sciences Laboratory, Research Triangle Institute, Research Triangle Park (N. Carolina 27709, USA), 10 July 1967.

[11] Gel permeation chromatography on polystyrene-divinyl benzene polymers has been applied chiefly to polymer mixtures [J. C. Moore, J. Poly. Sci. *2A*, 835 (1964)]. An example of its application to lipids has been recorded by C. L. Tipton, J. W. Paulis and M. D. Pierson, J. Chromat. *14*, 486 (1964). We thank Dr. Tipton for helpful correspondence.

[12] A report of work done under contract with the U.S. Department of Agriculture and authorized by the Research and Marketing Act of 1946. The contract is being supervised by the Eastern Utilization Research and Development Division of the Agricultural Research Service. Mass spectra were obtained by Dr. Maurice Bursey of the University of North Carolina.

Chemical Investigation of *Pluchea lanceolata* I. Isolation of a New Quaternary Base, Pluchine

Pluchea lanceolata, Linn. (N.O. Compositae) (Sanskrit, Hindi, Marathi: *Rasna*; Gujerati: *Rashna*; Bombay: *Kura*, *Sanna*) is a small shrub growing wild in the hotter parts of India, and is used in the Ayurvedic system of medicine in various clinical conditions. It is used as a bitter, a laxative, an analgestic, an antipyretic and a nerve tonic, and for the treatment of rheumatism, dyspepsia and bronchitis[1]. Preliminary pharmacological investigations with the water-soluble fraction of the ethanolic extract of the whole plant of *P.* lanceolata was done by Prasad et al.[2,3]. Detailed chemical investigation of *P. lanceolata* was, therefore, undertaken to isolate the active principles. The petroleum ether extract of *P. lanceolata*, on saponification with strong alkali, extraction with ether, chromatography on aluminium oxide (Brockmann) by elution with different solvents, yielded the following compounds: (1) Compound A, m.p. 92–93 °C (small yield). (2) Compound B, long needles, m.p. 217–221 °C, $[\alpha]_D^{20}$ + 89.2 ($CHCl_3$). Analysis[4] found: C, 83.79, 84,05; H, 11.52, 11.60. Calc. for $C_{30}H_{50}O$: C, 84.44; H, 11.81. *Acetate*, m.p. 238–242 °C, $[\alpha]_D^{20}$ + 91.5, + 95.2 ($CHCl_3$). Analysis found: C, 83.79; H, 11.31. Calc. for $C_{30}H_{49}O.CO.CH_3$: C, 82.05; H, 11.11.

In the Liebermann-Burchard colour reaction, the interface turned violet to brown, and chloroform layer turned pink on standing. No blue or green shade was obtained, indicating that compound B is not a sterol but a triterpenoid. Compound B has no UV-absorption between 220–340 nm, and has main IR-absorption peaks (Nujol) at 3.05 (m), 9.6 (m), 10.3 (m) and 11.42 (S) microns (Figure 1, sample V-25-1)[5]. It is probably Taraxasterol[6].

(3) Compound C, m.p. 147–150 °C, $[\alpha]_D^{20}$ – 27.25, – 26.05 ($CHCl_3$). Analysis found: C, 84.06, 84.15; H, 11.54, 11.74; mol. wt. 374, 362. Calc. for $C_{29}H_{50}O$: C, 83.99; H, 12.15; mol. wt. 414.69. *Acetate*, m.p. 130–132 °C, $[\alpha]_D^{20}$ – 47.5 ($CHCl_3$). Analysis found: C, 81.39, 81.61; H, 10.86, 10.76; mol. wt. 385. *Benzoate*, m.p. 149–150 °C; $[\alpha]_D^{20}$ – 19.68 ($CHCl_3$). Analysis found: C, 83.59, 83.43; H, 10.17, 10.19; mol. wt., 443. Compound C has no UV-absorption between 220–340 nm. It has main IR-absorption peaks (Nujol) at 2.98 (S), 9.42 (S), 9.77 (m), 10.32 (m) and 12.5 (m) μ (Figure 2, sample V-24-1), gives a positive Liebermann-Burchard reaction (blue to green), and is probably γ-sitosterol[7].

The total steroidal and terpenoidal fraction has been found by Prasad[8] to have no significant antiinflammatory activity when tested by the carragenin method.

After petroleum ether extraction, *P. lanceolata* was extracted exhaustively with ethanol by cold percolation. The solvent free extract was extracted with dilute hydrochloric acid. The acidic extract was found to contain very little tertiary bases, as tested by Mayer's reagent and paper chromatography, but contained considerable amounts of water-soluble quaternary bases which were precipitated by ammonium reineckate. The base reineckates were dissolved in acetone, decomposed with silver

[1] V. N. Dwivedi, *Bhavaprakash Nighantu* (Hindi translation) (Motilal Banarsi Das, Banaras, India 1949), p. 52; R. N. Chopra, I. C. Chopra, K. K. Handa and L. D. Kapur, *Indigenous Drugs of India*, 2nd edn (U.N. Dhur and Sons Ltd., Calcutta, India 1958), p. 520; K. R. Kirtikar and B. D. Basu, *Indian Medicinal Plants* (L. M. Basu, Allahabad, India 1933), vol. 2, p. 1345.

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[3] D. N. Prasad, S. K. Bhattacharya and P. K. Das, Indian J. med. Res. *54*, 582 (1966).

[4] All microanalyses were carried out by Dr. G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England.

[5] The author is indebted to Dr. M. N. Mitra, St. Louis, Missouri, USA for all UV- and IR-spectra carried out respectively in Cary's Spectrophotometer (in ethanol) and in Model 21 Double Beam Infra Red Spectrophotometer, Perkin Elmer Corporation, USA in Nujol mull (1X, 5X – ordinary and expanded Scales).

[6] E. H. Rodd, *Chemistry of Carbon Compounds* (Elsevier Publishing Company, Amsterdam – New York 1953), vol. 2, part B, p. 730.

[7] *Elsevier's Encyclopaedia of Organic Chemistry* (Elsevier Publishing Company, Inc. New York – Amsterdam 1940) 14S, 1803.

[8] D. N. Prasad, unpublished report.