

NEW CHEMICAL CONSTITUENTS IN TOBACCO

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WITH a view to understanding the role of tobacco in the production of oral cancer,¹ comprehensive study on the statistical, clinical, biological and chemical aspects of this problem were initiated at this Institute in 1950. Work on the statistical² and clinical^{3,4,5} aspects of the problem was undertaken first while that on the latter two aspects of the problem was started later on in 1953. Since then, isolation of γ -sitosteryl glycoside in tobacco⁶ and biological effects of the tobacco extracts on experimental animals⁷ have been reported. Additional studies on the chemistry of tobacco,* particularly with reference to the new constituents in it, are presented in this paper.

Although a wealth of information on the chemistry of tobacco⁸⁻¹⁰ is available, still it is far from complete. There is ample scope for isolation of tobacco constituents occurring in very small quantities, by employing newer techniques. In the present studies, we have attempted to isolate the constituents of a sun-cured variety of tobacco (*N. tabacum* known as 'Meenam-palayam' in the Coimbatore district) through chromatography of its extracts on alumina column. The process was rendered very laborious on account of a number of constituents in each extract, but the labours were ultimately rewarded in that some of the tobacco constituents which had eluded isolation by other methods were separated in the course of these studies.

The tobacco was successively extracted with petroleum ether (I), ether (II), benzene (III), chloroform (IV) and alcohol (V). No chemical work could be done on the extracts (III and IV) as they were used in entirety for biological work. Chromatographic fractionation of the tobacco extracts (I, II and V) is given below.

* A major part of the chemical work was submitted for the Ph.D. Degree (1957) by Mr. V. D. Divekar, and the remaining part was submitted towards the partial fulfilment of M.Sc. Degree (1959) by Mr. R. Y. Ambaye to the University of Bombay.

FRACTIONATION OF THE PETROLEUM ETHER EXTRACT

The petroleum ether extract was passed through an alumina column prepared in petroleum ether and the chromatogram was fractionated by elution with different eluents into three parts: I-A, I-B and I-C.

Isolation of paraffin hydrocarbons and waxy compounds containing oxygen, from I-A.—The eluate (I-A) on concentration deposited a solid which on crystallization from alcohol separated into a sparingly soluble compound, m.p. 84.5° and a rather freely soluble compound, m.p. 81.5° . The clear filtrate from the concentrate of (I-A) was chromatographed on alumina and eluted with petroleum ether. The eluates were collected by following the fluorescence of the bands on the chromatogram in ultra-violet light (Fig. 4). The eluates on vacuum distillation yielded four oily fractions which solidified on standing and on crystallization melted at 60° , 64.5° , 68° and $70-71^\circ$. The compound, m.p. 68° , occurring in major quantity from its properties and infra-red spectrum (Fig. 1) was identified as hentriacontane, $\text{H}_3\text{C}-(\text{CH}_2)_{29}-$

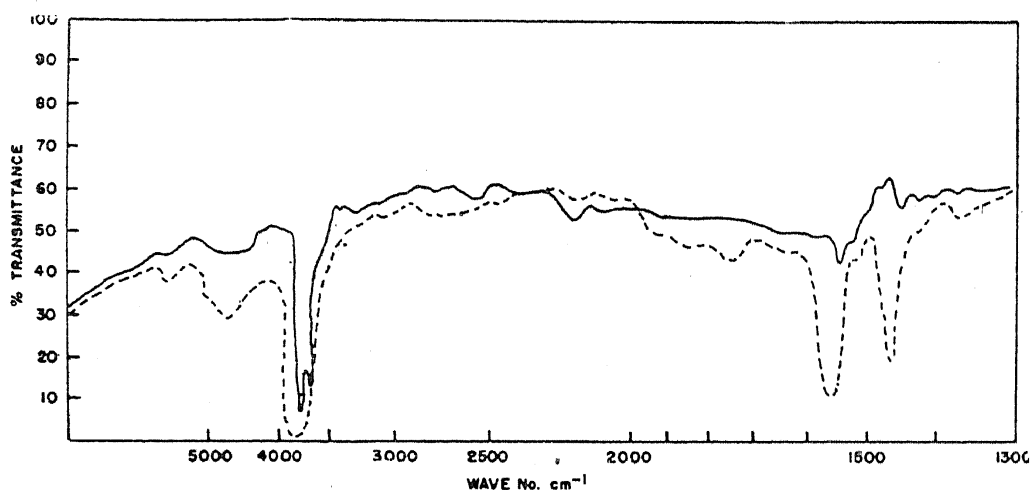


FIG. 1. Infra-red spectra of hentriacontane from petroleum ether extract of Meenampalayam tobacco (in CCl_4) and *n*-hexane. — Hentriacontane. - - - *n*-hexane.

CH_3 already reported^{11, 12} to be occurring in tobacco. The properties of compounds, m.p. 64.5° and m.p. 60° , indicated them to be lower homologues of hentriacontane, *viz.*, nonacosane $\text{CH}_3-(\text{CH}_2)_{27}-\text{CH}_3$ and heptacosane $\text{CH}_3-(\text{CH}_2)_{25}-\text{CH}_3$. The latter compound has been isolated^{11, 13} from tobacco but the former one seems to have escaped isolation so far. In the present studies both hentriacontane and nonacosane appeared in the initial eluates during chromatography of the ethereal and alcoholic extracts of the tobacco. The compound, m.p. $70-71^\circ$, however, could not be identified as it occurred in very small quantity. The eluate, obtained on further eluting

the chromatogram with mixture of petroleum ether and benzene (90:10), yielded a solid which on crystallization from alcohol separated into two fractions less soluble (m.p. 76–77°) and insoluble (m.p. 79°). The column was further eluted with a mixture of petroleum ether and benzene (80:20) when a compound, m.p. 76.5°, was obtained from the eluate. Finally, the column was eluted with alcohol and the alcoholic eluate yielded a compound, m.p. 74°. The compounds, m.ps. 84.5°, 81.5° and 76–77°, contained oxygen as was evident from their repeated carbon-hydrogen analysis and the absence of tests for other elements. On boiling with acids and alkalis the compounds remained unaffected; they did not undergo change on treatment with potassium permanganate or chromic acid. They did not form O-acetyl or ketonic derivatives. The compounds have, therefore, been tentatively classified as oxygen containing waxy compounds. Compounds, m.ps. 79°, 76.5° and 74°, were obtained in traces and were insufficient for characterisation.

A study of the carotenoid fraction (I-B).—The eluate (I-B) was concentrated and chromatographed on alumina column, when a bright orange-red band appeared on the column. On eluting the column with a mixture of petroleum ether and benzene (80:20) the band moved down. It was twice purified by chromatography. It was epiphasic in nature. It gave Carr-Price reaction and characteristic ultra-violet absorption peaks (426, 456 and 480 m μ) in ethyl alcoholic solution for β -carotene. The presence of β -carotene was thus confirmed in the extract.

Sterols from the fraction (I-C) after saponification.—The extract (I-C) was made alkaloid-free and saponified with alcoholic potassium hydroxide. The non-saponifiable part of the hydrolysate yielded two sterols, one m.p. 157° and another m.p. 165°. These sterols were also obtained from the tobacco extracts (II and V) and would be described in the later part of this paper.

FRACTIONATION OF THE ETHER EXTRACT

The ether extract, on cooling, sedimented a granular material, m.p. 225–50°. The solution remaining after separation of the material was evaporated and a residue (S) was obtained. Fractionation of the material, m.p. 225–250°, and the residue (S) would be discussed separately.

Separation of steryl glycosides from the sediment, m.p. 225–50°.—The granular material, m.p. 225–250°, on crystallization from alcohol separated into two fractions; one sparingly soluble and another freely soluble. Repeated crystallization of these two fractions from alcohol yielded four

compounds possessing melting points (A) m.p. 285°, (B) m.p. 287°, (C) m.p. 293° and (D) m.p. 295°. All the four compounds were laevorotatory. They formed tetra-acetyl derivatives which on saponification regenerated the original compounds. The tetra-acetates also were laevorotatory. The four compounds on hydrolysis with acid yielded sterols of different melting points with a common sugar moiety, *d*-glucose identified through its characteristic glucosazone, m.p. 208°.

Separation of a ketosteroid and sterols from the residue (S).—The residue (S) was taken in benzene and the solution chromatographed on alumina column. The chromatogram was developed and eluted with petroleum ether. Only the eluate (II-A), appearing colourless in ultra-violet light was collected. This eluate yielded nonacosane. The column was then eluted with mixtures of petroleum ether and benzene in increasing proportion of benzene and the eluates exhibiting characteristic fluorescence under ultra-violet light were pooled (II-B). (II-B) yielded a colourless, crystalline compound, m.p. 263–64°. The compound had a molecular weight 431, determined by the X-ray crystallographic method.* Carbon-hydrogen analysis indicated for it the empirical formula $C_{30}H_{52}O$. A molecular formula $C_{30}H_{52}O$ was, therefore, assigned to the compound. The compound formed 2:4-dinitrophenylhydrazone, m.p. 296° C. The compound, m.p. 263–64°, exhibited characteristic absorption bands in the finger-print region for steroids in its infra-red spectrum (Fig. 2) and showed a peak at 1700 cm^{-1} characteristic of the ketonic group. The compound has thus been confirmed as ketosteroid.

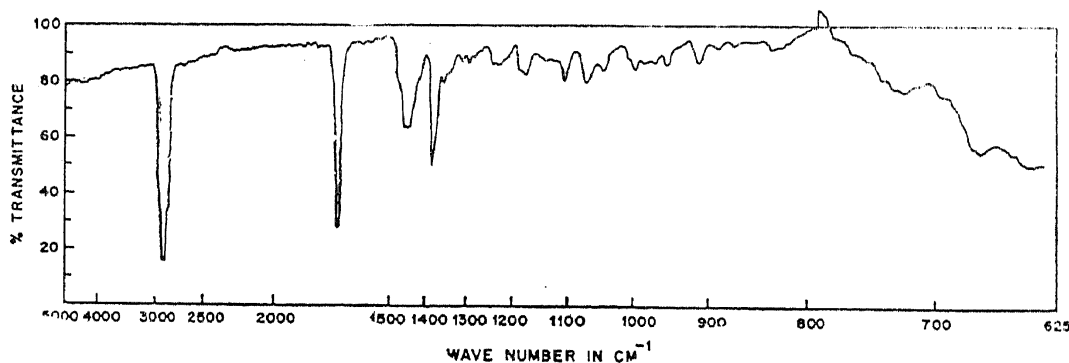


FIG. 2. Infra-red spectrum of ketosteroid, m.p. 263–64°, in $CHCl_3$.

Further elution of the chromatographic column was done with a mixture of petroleum ether and benzene or benzene and chloroform in different proportions. All the eluates were pooled (II-C) and subjected to rechromato-

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graphy. On elution with benzene-chloroform mixtures in increasing proportion of chloroform, eluates were obtained which yielded sterols, m.ps. 165°, 157°, 144° and 136·5°. These sterols were found to be identical by mixed melting points with the sterols obtained on hydrolysis of the glycosides, m.ps. 285° 287°, 293° and 295° respectively. A modified method was used for the hydrolysis of glycosides.^{14,15}

During the chromatography of sterols, it was noted that they were not eluted with either petroleum ether or benzene but were eluted only on addition of chloroform to benzene. It was also noted that the low melting sterols were eluted first and with less proportion of chloroform in the eluent mixture (benzene-chloroform); while higher melting sterols required higher proportion of chloroform in the eluent mixture. The sterols were laevorotatory in chloroform solution. They formed mono-acetates and mono-benzoates. They gave Liebermann-Burchard test and indicated unsaturation in the 5:6-position in the steroid skeleton. They exhibited peaks in the finger-print region 800–1500 cm^{-1} and 1550 cm^{-1} for steroids, and indicated the presence of a double bond in them (Fig. 3). The occurrence of sterol, m.p. 157°, and of the glycoside of sterol, m.p. 144° in alcohol extract, the presence of sterols, m.ps. 157° and 165°, in the non-saponifiable portion of the petroleum ether extract and identity of all these sterols with those occurring in the ether extract indicated the purity of individual sterols.

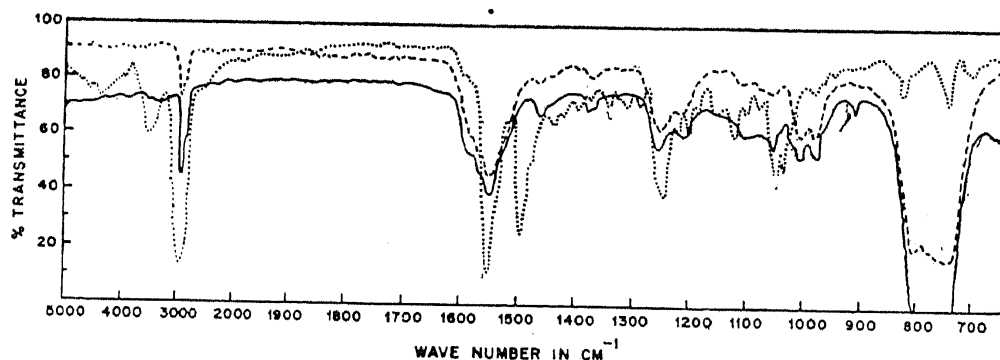


FIG. 3. Infra-red spectra of sterols in CCl_4 . ···· Steviol, m.p. 144°. ----- Sterol, m.p. 157°. ——— Sterol, m.p. 165°.

Sterols and their characterisation.—Sterol, m.p. 136·5° ($\text{C}_{29}\text{H}_{50}\text{O}$) from its properties appeared to be a β -sitosterol, commonly found in plants.¹⁶ It was characterised through its acetate and benzoate. The constitution of its glycoside, m.p. 295°, therefore, followed as β -sitosteryl-*d*-glucoside.

Sterols, m.p. 157° and m.p. 144°.—Ultimate analysis of these two sterols indicated that they contained half a molecule of solvent of crystallization,^{17,18}

methyl alcohol (for similar observation in the case of sterols *vide* Heilbron *et al.*,¹⁸ and King and Jurd¹⁷). Based on this assumption the sterols, m.ps. 157° and 144°, appear to be of the molecular formulæ $C_{28}H_{48}O$ and $C_{29}H_{50}O$ respectively.

Sterol, m.p. 165°.—Carbon-hydrogen analyses of this sterol and of its derivatives fit in very well for a formula $C_{27}H_{44}O$ as shown below:

CHART I

Compound m.p.	Found		Calculated for	C%	H%
	C%	H%			
Glycoside, 285°	72.1	9.5	$C_{33}H_{54}O_6$	72.49	9.96
Gly. tetra-acetate, 140°	69.3	9.0	$C_{41}H_{62}O_{10}$	68.88	8.74
Sterol, 165°	84.3	11.8	$C_{27}H_{44}O$	84.31	11.53
St. acetate, 143°	81.3	10.9	$C_{29}H_{46}O_2$	81.63	10.87
St. benzoate, 164°	84.2	10.2	$C_{31}H_{48}O_2$	83.55	9.9

C_{27} -sterol is rather unusual in plant sterols and thus appears to be of interest.

FRACTIONATION OF THE ALCOHOL EXTRACT

The alcoholic extract of tobacco was concentrated to a thick paste and the slurry poured in water when it separated into water-soluble and water-insoluble fractions. The water-insoluble part was treated with benzene and further resolved into benzene-soluble (V-A) and benzene-insoluble (V-B) parts. The benzene insoluble (V-B) part on extraction with ether yielded a glycoside, m.p. 292–294°, identical with the one found in the ether extract. On hydrolysis it gave a sterol, m.p. 144–45°, identical by mixed melting point with the sterol of the same melting point obtained from the petroleum ether extract or from the glycoside, m.p. 293°, occurring in the ether extract. The benzene-soluble part (V-A) on chromatography over alumina column and elution with petroleum ether yielded a mixture of easily oxidisable oils and hentriacontane. Further elution of the column with benzene yielded a free sterol, m.p. 157°, identical with the sterol of the same melting point occurring in the ether extract, and oxidisable oils. Final elution with ethyl alcohol gave light-coloured oil which on standing turned reddish-brown in colour and was susceptible to air-oxidation.

EXPERIMENTAL

A sun-dried variety of tobacco (*N. tabacum*), locally called 'Meenam-palayam' in Coimbatore district (South India), was procured in the form of hangs of dark-brown leaves. The leaves contained on an average 10–12% of moisture. The leaves were dried at about 60° under current of hot air in an oven and powdered (80 mesh). The powder was stored in well-packed containers to avoid any fungal growth.

Solvent extraction of tobacco.—The tobacco powder was exhaustively extracted in a soxhlet extractor or on a large scale in a modified extraction apparatus¹⁹ with (I) petroleum ether (b.p. 60–80°), (II) ether, (III) benzene, (IV) chloroform and (V) ethyl alcohol in succession. The solvents of extraction were completely removed and the residues were weighed. The average petroleum ether extractives amounted to 8.6%, ether extractives 1.32%, benzene extractives 6.8%, chloroform extractives 3.97% and alcohol extractives 12.35% of the dried material, the total being 33.04%.

Chromatographic fractionation of the petroleum ether extract.—Petroleum ether extract (4% solution) was passed through a column of alumina (Merck, Analytical Grade, 120 mesh) in petroleum ether. The chromatogram was developed with petroleum ether, when two zones were visible in the upper part of the column, in the ordinary light. A third zone, at the bottom of the column, consisting of five bands of blue and yellow colour strips close to each other, became visible when the column was exposed to ultra-violet light (Fig. 4). On eluting the chromatogram with petroleum ether, colourless eluate (I-A) consisting of the bottom zone was obtained. The eluate did not react with alkaloidal reagents. The column was then eluted with a mixture of petroleum ether and benzene (80 : 20) when an orange-coloured eluate (I-B), that formed the middle zone, was obtained. Lastly, the column was exhaustively eluted with benzene, chloroform and alcohol in succession, the solvents were removed from the three eluates under partial vacuum, and the residues combined (I-C). The three fractions I-A, I-B and I-C were then worked as follows:

Fractionation of Part I-A.—The eluate I-A on concentration yielded a colourless deposit which was separated by filtration and the clear filtrate (F) was reserved for further study. The solid deposit was exhaustively treated with warm alcohol when it separated into two portions—one soluble and another insoluble in alcohol. The insoluble part remained in molten state during treatment with boiling alcohol and solidified on cooling. The alcohol-soluble portion crystallised on cooling in plates, m.p. 81.5° (Found : C, 81.8; H, 13.7; $C_{24}H_{48}O$, requires C, 81.74; H, 13.72%). Yield

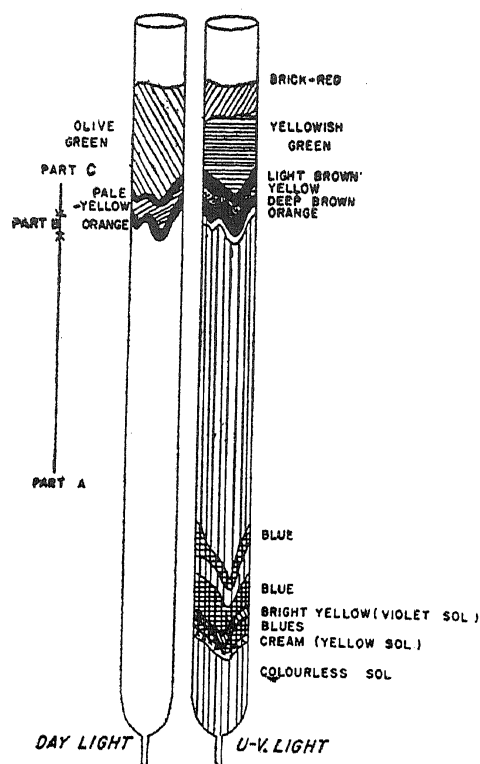


FIG. 4. Chromatographic fractionation of petroleum ether extract.

0.001%. The alcohol-insoluble compound crystallised from a mixture of benzene and alcohol (20:80) in plates, m.p. 84.5° (Found: C, 82.2; H, 14.0% $C_{29}H_{58}O$; requires C, 82.39; H, 13.83%). Yield 0.001%. A mixed melting point of the two compounds was depressed by about 5° . Both the compounds are insoluble in 10% sodium hydroxide solution and 5% sodium bicarbonate solution. They did not decolourise the potassium permanganate solution (2%).

The clear filtrate (F) remaining after the separation of compounds, m.ps. 81.5° and 84.5° , was chromatographed on alumina column. The chromatogram was developed with petroleum ether. The positions of the chromatographic bands appearing in ultra-violet light are shown in Fig. 4. The chromatogram was eluted with petroleum ether. The clear and non-fluorescent eluate was collected in three portions. From the eluates, three different waxy residues differing in melting points from $60-69^{\circ}$ were obtained on concentration. The waxy residues were mixed and distilled under vacuum when the following four fractions were obtained:

Fraction 1.—b.p. $160-65^{\circ}/0.01$ mm.: It crystallised from ethyl alcohol in plates, m.p. 60° . It was obtained in traces.

Fraction 2.—b.p. 165–70°/0.01 mm.: It was crystallised from ethyl alcohol in plates, m.p. 64.5° (Found: C, 85.6; H, 14.6, calculated for $C_{29}H_{60}$, C, 85.2; H, 14.8%). Yield, 0.001%.

Fraction 3.—(Hentriacontane), b.p. 197–200°/0.02 mm. : It crystallised from ethyl alcohol in plates, m.p. 68° (Found: C, 85; H, 15%; calculated for $C_{31}H_{64}$ C, 85.23; H, 14.77%). Yield 0.16%.

Fraction 4.—b.p. 240–50°/0.02 mm. : It crystallised from ethyl alcohol in plates, m.p. 70–71° (C, 83.0; H, 12.7%). Yield 0.05%.

None of the four compounds in carbon tetrachloride solution could decolourise 2% solution of bromine in carbon tetrachloride.

On continuing elution of the column with petroleum ether, liquids with characteristic fluorescence in ultra-violet light were obtained. The column was then eluted with ethyl alcohol. From the eluate, solvent was removed and the residue was taken in petroleum ether. The solution was chromatographed on alumina column. Chromatographic bands visible under ultra-violet light were eluted as under:

The part exhibiting violet fluorescence was eluted with petroleum ether. This on slight concentration deposited a solid which was filtered. The solid deposit was treated with warm alcohol several times, when it separated into two components one (m.p. 81.5°) alcohol-soluble and another (m.p. 79°) insoluble in alcohol. The latter component was obtained in traces. A mixed melting point of the former component with the component of similar melting point obtained from (I-A) was not depressed.

The column was then eluted with petroleum ether and benzene (90 : 10). The eluate had sky-blue fluorescence. On concentration, it deposited a solid, which was exhaustively treated with boiling alcohol when it separated into two components one soluble and another insoluble in alcohol. The alcohol-soluble component crystallized from the solvent in plates, m.p. 76–77° (C, 83.9; H, 12.9%). Yield 0.01%. The alcohol-insoluble component melted at 79° and was obtained in traces. The solutions of the compounds m.p.s. 79° and 76–77°, in carbon tetrachloride, did not decolourise 2% solution of bromine in carbon tetrachloride.

Elution with petroleum ether and benzene (80 : 20) gave an oil, which on concentration yielded a mixture of yellow-blue fluorescent oil and a small amount of waxy compound which crystallised from ethyl alcohol in plates, m.p. 76.5°.

The column was finally eluted with ethyl alcohol. The eluate was distilled under partial vacuum for removal of alcohol. The yellow residue left

behind was dissolved in boiling alcohol when a crystalline waxy compound, m.p. 74° , separated. Mother liquors of crystallization on concentration gave a yellow liquid.

Attempts to characterise compounds, m.ps. 84.5° , 81.5° , $76-77^{\circ}$ and 71° occurring in I-A.—Each one of these compounds (a few mg.) did not undergo (1) acetylation on boiling (for 24 hours) with acetic anhydride and a few drops of pyridine. (2) Formation of ketonic derivatives on boiling with 2 : 4-dinitrophenylhydrazine hydrochloride and a few drops of concentrated sulphuric acid (20 hours) or of an oxime, on boiling (20 hours) with hydroxylamine hydrochloride, did not take place. (3) They were not affected on boiling for 24 hours with sodium hydroxide or potassium hydroxide (30%), or with 10% sodium hydroxide or 10% sulphuric acid, or on heating in a sealed tube at 120° either with 20% either sodium hydroxide or 20% sulphuric acid. (4) They were recovered unchanged on boiling with 5% potassium permanganate or 75% chromic anhydride in acetic acid.

Separation of carotenoid from I-B.—The concentrate of I-B was taken in petroleum ether and chromatographed on alumina column when a prominent orange-red band was seen on the column. It moved down with a mixture of petroleum ether and benzene (80 : 20). The eluate was rechromatographed thrice when it was obtained as a bright orange liquid. The residue obtained on removal of the solvent gave the Carr-Price reaction for carotenoids.²⁰

Fractionation of I-C.—A portion of I-C was taken in chloroform. The chloroformic solution was shaken with 2% hydrochloric acid in a separating funnel for removal of alkaloids. Chloroform was removed from the alkaloid-free extract (I-C₁) and it was (1) studied for the different constituents in it by chromatography and (2) for the constituents in its non-saponifiable portion.

Fractionation of I-C₁.—A solution of the extract (I-C₁) in petroleum ether (about 2%) was chromatographed on alumina column. The chromatogram was developed with petroleum ether when a prominent greenish-yellow fluorescent band (between the upper and lower zones on the column) appeared on the chromatogram (Fig. 4).

The lower zone was composed of a number of narrow yellow bands interposed with blue bands (in ultra-violet light, Fig. 4), while the upper zone appeared dirty yellow (in daylight). Since it was difficult to separate the various bands in the lower zone by chromatographic elution, the column was extruded and the middle (fluorescent) band was cut from the upper and lower zones by a spatula. The three portions of the column were separately extracted with boiling alcohol and alcohol was removed from the extracts under partial

vacuum. The oily residues obtained in all these portions were separately rechromatographed on an alumina column for further purification, and they yielded different fluorescent oily fractions in very small amounts.

Constituents in the non-saponifiable portion of I-C₁.—A portion of I-C₁ was saponified by refluxing with 20% alcoholic potash on a water-bath for 1 hour. The solution was diluted with water and extracted with ether. The ether extract was washed with water and concentrated by distillation under reduced pressure. To the non-saponifiable portion (residue) methyl alcohol was added. The mixture was then refluxed for a while and the methyl alcoholic layer was decanted off. The non-saponifiable part was extracted several times with methyl alcohol. The methyl alcoholic washings were pooled and concentrated under partial vacuum when a colourless crystalline material was obtained. It gave Liebermann-Burchard test for sterols. On repeated crystallizations from methyl alcohol, it separated into two crystalline compounds melting at 156–57° and 165°. They were found to be identical with sterols of same melting points found in the ether extract.

Fractionation of I-C₂.—The extract, on vacuum distillation, yielded an oil, b.p. 130–40°/0.02 mm.

ETHER EXTRACT

The ether extract of tobacco on cooling deposited a colourless granular mass on the sides and bottom of the extraction flask. The granular material and the ether-soluble portion were worked up separately as given below:

Fractionation of the granular material, m.p. 225–50°.—The granular material, m.p. 225–50°, was dissolved in a fairly good amount of ethyl alcohol and allowed to crystallise. The sparingly-soluble product, that separated out on cooling, melted at 269–70°. The mother liquors of crystallization on concentration yielded a solid (A), which was again subjected to crystallization and by repeating this process as much of the compound, m.p. 269–70°, that could be recovered from the mother liquors was obtained. The compound, m.p. 269–70°, was then further purified through repeated crystallizations to a constant melting point product, m.p. 285°. The mother liquors of crystallization of the products, m.ps. 269–78° and 285°, were pooled, concentrated and processed in a manner described earlier, to obtain another *moderately-soluble* fraction, m.p. 287°. In a similar way, the mother liquors of crystallization of the product, m.p. 287°, yielded a third *fairly-soluble* fraction, m.p. 293°, and finally a *highly-soluble* product, m.p. 294–95°, was obtained from the mother liquors of the crystallization of the product,

m.p. 293°. The granular material, m.p. 225–50°, was thus fractionated by repeated crystallizations into four glycosides, with melting points 285°, 287°, 293° and 295°.

General properties of the glycosides.—The glycosides are insoluble in petroleum ether, benzene, chloroform and sparingly soluble in ether, acetone, alcohol and glacial acetic acid. They are insoluble in 10% sodium hydroxide solution and 10% sodium bicarbonate solution. They are resistant to hydrolysis with hydrochloric acid of less than 10% strength. The suspension of glycosides in chloroform showed a Liebermann-Burchard test giving a play of colours changing from pink, red to green. Aqueous suspension of the glycosides gave a Molische test.

Hydrolysis of the glycosides.—Glycoside (0.1 g.) was refluxed with 20% hydrochloric acid (10 c.c.) for 15–20 minutes. To this reaction mixture, petroleum ether (10 c.c.) was added and the mixture again refluxed for 2–3 minutes. On cooling the reaction solution, petroleum ether layer was separated by means of a separating funnel from the acidic layer. To the acidic layer which usually contained some insoluble matter, petroleum ether (10 c.c.) was added and the solution was again refluxed for 2–3 minutes. In this way 2–3 treatments were given to the acidic solution and the petroleum ether washings were pooled. The washings were washed first with 10% bicarbonate solution and then with distilled water. Removal of petroleum ether from the extract gave a crystalline aglycone. The aglycones obtained from the four glycosides gave Liebermann-Burchard test for sterol. They were soluble in petroleum ether, chloroform, benzene and sparingly soluble in alcohol, acetone and glacial acetic acid. The acidic layer of the hydrolysate was neutralised with sodium bicarbonate. The neutral solution (5 c.c.) yielded a brick-red precipitate of Cu_2O , on boiling with Fehling's solution (2 c.c.).

Preparation of glucosazones.—Hydrolysate (20 c.c. obtained from the hydrolysis of 0.1 g. of glycoside), neutralised with sodium bicarbonate, was taken in a flask and acidified with acetic acid. To the solution was added sodium acetate (1.0 g.) and colourless phenylhydrazine hydrochloride (1.0 g.). The mixture was refluxed on a water-bath for 40 minutes, diluted with water and kept in a refrigerator. The osazone separated as a fluffy yellow orange mass. It was filtered and washed first with dilute alcohol and then with distilled water. It crystallised from pyridine-water mixture (20 : 80) in orange-yellow needles. The osazones, m.p. 205–08°, from the 4 glycosides were found to be identical by a mixed melting point with that of the authentic

sample of osazone of *d*-glucose, which was prepared after the method described by Fischer.²¹

Tetra-acetates of the glycosides.—Glycoside (0.1 g.) was refluxed with acetic anhydride (10 c.c.) and pyridine (2 to 3 drops) for one hour. The mixture was then diluted with water, filtered and the solid material was washed with 2% hydrochloric acid and then with distilled water. The resulting tetra-acetate was crystallised from methyl alcohol several times until it showed constant melting point. The constants of the tetra-acetates obtained from four glycosides were as follows:—

Glycoside m.p.	Tetra- acetate m.p.	Optical rotation [α] _D ^{20°} in pyridine	Found		Analysis Calculated for	C%	H%
			C%	H%			
285°	140°	−46.12°	69.3	9.0	C ₄₁ H ₆₂ O ₁₀	68.88	8.74
287°	154–55°	−37.82°	69.1	9.2	C ₄₂ H ₆₆ O ₁₀	69.04	9.04
293°	159–60°
292–94° (via Alcohol extract)	159–60°	−33.18°	69.3	9.2	C ₄₃ H ₆₈ O ₁₀	69.3	9.1
295°	169.5°	−38.07°	69.4	9.2	C ₄₃ H ₆₈ O ₁₀	69.3	9.1

Regeneration of glycoside from the tetra-acetate.—The tetra-acetate (0.04 g.) was dissolved in alcohol (5 c.c.) and a solution of sodium hydroxide (5 c.c. of 10%) was slowly added to it. The mixture was refluxed for one hour on a water-bath and then allowed to cool in a refrigerator. The precipitate of regenerated glycoside was filtered, washed free of alkali, first with dilute acid and then with distilled water. It was crystallised from ethyl alcohol. The properties of glycosides are given below:

Tetra- acetate m.p.	Regene- rated glycoside m.p.	Optical rotation [α] _D ^{18°} in pyridine	Found		Analysis Calculated for	C%	H%
			C%	H%			
140°	285°	−62.55°	72.1	9.5	C ₃₃ H ₅₄ O ₆	72.41	9.89
154–55°	287°	−50.7°	71.9	10.3	C ₃₄ H ₅₈ O ₆	72.50	10.32
159–60° (via Alcohol extract)	292–94°	−54.88°	72.7	9.6	C ₃₅ H ₆₀ O ₆	72.80	10.4
169.5	295–96°	−54.08°	72.3	9.9	C ₃₅ H ₆₀ O ₆	72.80	10.4

Hydrolyses of glycosides were carried out under conditions described earlier for aglycones. They were crystallised from methyl alcohol until they showed sharp melting points. The yield and properties of different aglycones (sterols) are given below:

Glycoside		Sterol		Optical rotation [α] _D ^{18°} in chloro- form	Found		Analysis Calculated for	C%	H%
m.p.	% yield	m.p.	% yield		C%	H%			
285°	0.25	165-66°	0.16	-48.56°	84.30	11.8	C ₂₇ H ₄₄ O	84.31	11.53
287°	0.05	157-58°	0.03	-51.24°	83.0	11.8	C ₂₈ H ₄₈ O, $\frac{1}{2}$ CH ₃ OH	82.40	12.0
293°	0.06	144°	0.04	..	82.6	11.4	C ₂₉ H ₅₀ O, $\frac{1}{2}$ CH ₃ OH	82.31	11.9
292-94° (via Alcohol extract)	0.002	144-45°	0.001	-43.2°	82.6	11.4	C ₂₉ H ₅₀ O, $\frac{1}{2}$ CH ₃ OH	82.31	11.9
295°	0.04	136.5°	0.02	-45.77°	82.8	11.8	C ₂₉ H ₅₀ O, $\frac{1}{2}$ CH ₃ OH	82.31	11.9

Preparation of sterol acetates.—Sterol (0.1 g.) was refluxed for one hour on a wire-gauze with acetic anhydride (5.0 c.c.) and pyridine (2-3 drops). The mixture was diluted with water and cooled. The precipitate was filtered and washed first with 2% hydrochloric acid and then with distilled water. The acetates were crystallised from ethyl alcohol in plates. The properties of the acetates are given below:

Sterol m.p.	Sterol acetate m.p.	Optical rotation [α] _D ^{19°} in chloro- form	Found		Analysis Calculated for	C%	H%
			C%	H%			
165-66°	143°	-61.4°	81.3	10.9	C ₂₉ H ₄₆ O ₂	81.63	10.87
157-58°	138°	-51.2°	81.7	11.4	C ₃₀ H ₅₀ O ₂	81.39	11.38
144°	139°	..	82.7	11.6	C ₃₁ H ₅₂ O ₂	81.8	11.4
144-45° (via Alco- hol ext.)	138-39°	-54.05°	81.4	11.4	C ₃₁ H ₅₂ O ₂	81.7	11.4
136.5°	130°	..	82.3	11.8	C ₃₁ H ₅₂ O ₂	81.7	11.4

Preparation of sterol benzoates.—The sterol (0.02 g.), pyridine (2 c.c.) and benzoyl chloride (0.2 c.c.) were refluxed on a water-bath for one hour. The reaction mixture was then diluted with water and cooled. The separated benzoate was filtered, washed with 10% sodium bicarbonate solution and finally with distilled water. They crystallized from methyl alcohol. The properties of sterol benzoates are shown in the following table:

Sterol m.p.	Sterol benzoate m.p.	Optical rotation [α] _D ^{23.5°} in chloro- form	Found		Analysis	C%	H%
			C%	H%	Calculated for		
165–66°	164–65°	–35.0°	84.2	10.2	C ₃₄ H ₄₈ O ₂	83.50	9.9
157–58°	155–56°	..	83.3	10.4	C ₃₅ H ₅₂ O ₂	83.28	10.38
144–45° (via Alcohol ext.)	147–48°	–31.6°	82.6	9.7	C ₃₆ H ₅₄ O ₂	83.34	10.4
136.5°	140°	..	81.2	9.6	C ₃₆ H ₅₄ O ₂	83.34	10.4

Chromatographic fractionation of the ether extract remaining after the separation of the granular material, m.p. 225–50°.—The solvent was removed from the extract and the residue (S) was taken in benzene when a part of the material dissolved in benzene and a sticky mass remained as insoluble matter. The insoluble material was rejected. The benzene-soluble portion was chromatographed on alumina column. The column was eluted with six solvents. On elution with petroleum ether (1) a colourless non-fluorescent eluate was obtained. On concentration it yielded a waxy material (Nonacosane) which crystallised from ethyl alcohol in colourless plates, m.p. 64.5°. Yield 0.002% (Found: C, 85.6; H, 14.6%; calculated for C₂₉H₆: C, 85.2; H, 14.8%).

Elution of the chromatogram was continued with (1) and subsequently followed by (2) petroleum ether-benzene (90 : 10), (80 : 20) and (70 : 30) and (3) benzene alone. The eluates thus obtained were pooled separately. The column was then developed with (4) mixtures of benzene-petroleum ether (80 : 20, 90 : 10) in succession and eluted with chloroform-benzene mixture (10 : 90). The eluate yielded a yellow oil, which contained a crystalline sterol. It crystallised from methyl alcohol in needles, m.p. 138–40°. However, on repeated crystallizations from methyl alcohol, it separated into

two pure sterols of m.ps. 136° and 144°. The column was further developed with (5) mixture of benzene-chloroform (80 : 20) and eluted with benzene-chloroform mixture (70 : 30). The eluate yielded a pale-yellow oil, fluorescent (violet) on the column. This eluate gave crystalline sterol which crystallised from methyl alcohol in needles, m.p. 154–55°. On recrystallization from methyl alcohol, a pure sterol, m.p. 157°, was obtained. Finally, the column was developed with (6) mixtures of benzene-chloroform (50 : 50, 40 : 60) and eluted with chloroform alone. The eluate yielded a viscous oil and a crystalline sterol. The sterol crystallised from methyl alcohol in needles, m.p. 160–62°. On recrystallization from methyl alcohol, it melted at 165°.

The sterols, m.ps. 136°, 144°, 157° and 165°, obtained from eluates 4, 5 and 6 when mixed with sterols of the corresponding melting points, obtained from the glycosides, found in the ether extract showed no depression in melting points.

The solvents from the fractions (1), (2) and (3) were removed under reduced pressure and the residues were separately taken in chloroform and treated with 2% hydrochloric acid. From the alkaloid-free extract, chloroform was removed and the residues were taken in petroleum ether and separately rechromatographed on almuina column. The chromatograms were eluted with petroleum ether and benzene. The eluates (1 *a*), (2 *a*) and (3 *a*) yielded a crystalline compound, m.p. 263–64°, identical by mixed melting point. The compound crystallised from alcohol in colourless needles (Found: C, 84.1; H, 11.9%; $C_{20}H_{52}O$ requires C, 84.04; H, 12.23%) $[\alpha]_D^{20} = -28.7^\circ$ in chloroform.

2:4 Dinitrophenylhydrazone of the compound, m.p. 263–64°.—Hydrazone of the compound, m.p. 263–64° (15 mg.), was prepared by the usual method. It crystallised from ethyl acetate in beautiful long orange needles, m.p. 296–96.5° (Found: C, 71.7; H, 8.6, N, 8.9%; $C_{36}H_{56}O_4N_4$ requires C, 71.01; H, 9.27; N, 9.2%). Yield 0.017 g.

Attempt at the regeneration of the compound, m.p. 263–64°, from its hydrazone.—2:4-Dinitrophenylhydrazone (10 mg.), m.p. 263–64°, was heated on a steam-bath with 80% formic acid (25 c.c.) and freshly prepared copper carbonate for 3 hours.²² At the end of the reaction, the hydrazone was recovered unchanged.

ALCOHOL EXTRACT

Separation of the alcohol extract into water-soluble and water-insoluble portions.—Alcohol extract (10 lb.) of the tobacco was concentrated to a thick

paste and poured into distilled water (500 c.c.). The *water-soluble portion* (1) was extracted with ether and ether extract was separated into acidic, basic and neutral fractions. The basic fraction showed the presence of alkaloids. The acidic fraction gave test for organic acids. The *water-insoluble portion* (2) was dried, extracted with benzene and separated into benzene-soluble (V-A) and benzene-insoluble (V-B) parts. Part (V-B) was extracted with ether to obtain (V-B₁), which on concentration gave a compound, m.p. 270–80°. The ether-insoluble part (V-B₂) was rejected.

Separation of benzene-soluble part (V-A).—A concentrated solution of (V-A) was passed through a column of alumina. The chromatogram was developed with petroleum ether, when a colourless band with parrot-green fluorescence (U-V. light) was visible. It was eluted down with petroleum ether, *Part A'*. The chromatogram was then exhaustively eluted with benzene, *Part B'*, which consisted of a yellowish-green band and showed a greyish fluorescence (U-V. light). Final elution with ethyl alcohol yielded an orange band, *Part C'* with dirty-grey fluorescence in ultra-violet light.

Rechromatography of Parts A', B' and C'.—*Part A'* was concentrated and rechromatographed on a fresh alumina column, and a band giving parrot-green fluorescence (U-V. light) was eluted down with petroleum ether. On removal of the solvent from the eluate, yellow waxy residue with a strong fluorescence (U-V. light) was obtained. The residue was repeatedly extracted with boiling ethyl acetate and filtered while hot. On cooling, a colourless compound, m.p. 67.5–68°, in shining needles separated out. It was identified by mixed m.p. as hentriacontane, isolated from petroleum ether extract of tobacco. The mother liquor of crystallization yielded another compound, m.p. 37–39°. *Part B'* (from 100 lb. of tobacco) was concentrated under vacuum and kept in the refrigerator for over 6 months, when a crystalline compound appeared in the solution. The solution was extracted with boiling methyl alcohol (norit) and filtered. The crystalline compound obtained on cooling was separated and repeatedly crystallised from methyl alcohol when it melted at 157°. Yield 0.05 g. (Found: C, 82.6; H, 11.8% calculated for C₂₈H₄₈O, $\frac{1}{2}$ CH₃OH, C, 82.21; H, 12.0%).

Acetate of sterol, m.p. 157°.—m.p. 139° (methyl alcohol) (Found: C, 81.7; H, 11.0% calculated for C₃₁H₅₂O₂: C, 81.6; H, 11.4%) [α]_D¹⁹ — 52.0° (chloroform).

Parts B' and C' on vacuum distillation yielded the following unstable oils in very small quantities. They showed the presence of alkaloids and were found to contain N, S and halogens.

Part B': (i) A canary yellow oil, b.p. 75–80°/0·01 mm.

(ii) An orange liquid, b.p. 110–15°/0·01 mm.

(iii) A brown oil, b.p. 195–200°/0·03 mm.

Part C': (i) A pale yellow liquid, b.p. 65–70°/0·015 mm.

(ii) An orange viscous liquid, b.p. 150–55°/0·015 mm.

Isolation of sterol glycoside.—The ether extract (V-B₁) on concentration yielded a green liquid and a white granular material remaining at the bottom of the flask. The entire material was taken in boiling ethyl alcohol (norit) and filtered. On concentration of the filtrate, the white granular material separated out. It was filtered and crystallised from ethyl alcohol. The glycoside, thus obtained, melted between 270° and 280° (bath preheated to 270°). Yield 0·002%. This glycoside on crystallization from ethyl alcohol melted at 292–94° and did not show depression in melting point when mixed with the glycoside, m.p. 293°, found in ether extract. Yield 0·001%.

SUMMARY

New chemical constituents in a variety of Indian tobacco (*N. tabacum*) have been isolated by means of chromatography on alumina. The separation of four steryl glycosides, four sterols, one ketosteroid and nine waxy compounds have been reported in this paper. Attempts have been made to elucidate the chemistry of all these products.

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