kept at room temperature for 8 days, at 60° for 2.5 days and at 80° for 21 hours. When the reactants were refluxed in toluene at 110° for 12 hours, or in bromobenzene at 155° for 11 hours, there was considerable tar formation, less than 5% of the original sulfonyl fluoride could be recovered, and no adduct could be isolated.

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Autoxidation of Cholesterol during Purification *via* the Dibromide[1]

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RECEIVED JANUARY 25, 1954

Recent investigation of cholesterol biogenesis using carbon-14 has accentuated the problem of obtaining pure sterol preparations from animal tissues. Isolation as the sterol digitonide followed by conversion to the dibromide and subsequent reduction to the sterol have been advocated as reliable procedures for radiochemically pure cholesterol obtained from isolated organ perfusion and from intact animal experiments.[2] These procedures are not without objection in that artifacts may arise during decomposition of the digitonides and during bromination–debromination.[3]

In addition to these difficulties autoxidation of cholesterol may occur during the debromination reaction. In the routine use of the modified zinc–acetic acid debromination procedure of Schwenk and Werthessen[2a] the presence of several unidentified Liebermann–Burchard positive materials was discovered in supposedly pure preparations. These materials manifested themselves as characteristic patterns of spots on paper chromatograms, as recorded in Table I. In every instance spots corresponding to cholesterol and to the epimeric 7-hydroxycholesterols were found. Of the unidentified spots spot IV (system A) and spot III (system B) appeared in most of the chromatograms and were intense in some. Spot I in both systems occurred in most of the patterns and must correspond to higher oxygenated sterols. It is likely that spots I, IV and II of system A correspond to spots I, III and IV of system B, respectively.

The identities of Table I are assigned on evidence of relative migration on the chromatograms of the known and the suspect sterol, separately and in admixture, and on evidence of color behavior with the Liebermann–Burchard and antimony trichloride reagents. It should be emphasized that these evidences are from two independent paper chromatographic systems; the system of Neher and Wettstein[4] (system B) involves partition between two liquid phases, while the simple ascending system (system A, *o*-dichlorobenzene) does not. By examination of the debromination products in the two systems several sterols of possible implication are eliminated from consideration. Mobility data for related sterols are presented in Table II.[5]

TABLE I

CHROMATOGRAPHIC PATTERN OF DEBROMINATION REACTION PRODUCTS

Spot	Mobility[a]	Color behavior with[b] Liebermann–Burchard	$SbCl_3$	Tentative identity
System A (*o*-dichlorobenzene)				
I	0.08	Gr-G	Bl	.....
II	.32	G	..	.....
III	.61	Rs → V → Bl	Bl	Δ^5-Cholestene-3β,7-diols
IV	.84	P → Bl-G	Bl	.....
V	.93	V → Bl-G	V	Cholesterol
System B (β-phenoxyethyl alcohol–heptane)				
I	0.01	Gr-G	Bl	.....
IIA	.11	Rs → V → Bl	Bl	Δ^5-Cholestene-3β,7β-diol
IIB	.17	Rs → V → Bl	Bl	Δ^5-Cholestene-3β,7α-diol
III	.40	P → Bl-G	Bl	.....
IV	.53	P → Bl-G	..	.....
V	1.00	V → Bl-G	V	Cholesterol

[a] The mobility in system A (*o*-dichlorobenzene) is expressed in R_f; for system B (β-phenoxyethyl alcohol–heptane) the mobility is expressed in R_c. [b] Bl = blue, Rs = rose, G = green, V = violet, Gr = gray, P = pink. These colors and sequences are reproducible.

Control chromatograms on the original cholesterol and on the cholesterol dibromide employed were run concurrently with the debromination products; in each control chromatogram only the one component was present. Cholesterol treated with zinc and acetic acid and extracted in the exact manner employed in the debromination reaction did not contain autoxidation products. Cholesteryl acetate dibromide treated in like manner also failed to give the pattern of spots expected of cholesterol dibromide; only two components were detected, corresponding to cholesterol and to cholesteryl acetate.[7]

Patterns for debromination products using other debrominating agents such as sodium iodide–ethanol,[8] potassium acetate–ethanol[9] and sodium acetate–aqueous ethanol[9,10] were also complex. Although this line was not developed further, it is likely that the 7-hydroxycholesterols contribute to these patterns.

(1) Supported in part by funds from the Texas Heart Association and by funds under contract AF 18(600-)303 with the USAF School of Aviation Medicine, Randolph Field, Texas.

(2) (a) E. Schwenk and N. T. Werthessen, *Arch. Biochem. Biophys.*, **40**, 334 (1952); (b) **42**, 91 (1953).

(3) (a) G. A. D. Haslewood, *Biochem. J.*, **33**, 709 (1939); (b) K. Tsuda and B. Umezawa, *J. Pharm. Soc. Japan*, **71**, 273 (1951); (c) K. Tsuda, R. Hayatsu, B. Umezawa and T. Nakamura, *ibid.*, **72**, 182 (1952); (d) E. Schwenk, N. T. Werthessen and H. Rosenkrantz, *Arch. Biochem. Biophys.*, **37**, 247 (1952).

(4) R. Neher and A. Wettstein, *Helv. Chim. Acta*, **35**, 276 (1952).

(5) The mobilities in the paper partition system (system B) in Table II for the epimeric pairs: 7α-hydroxycholesterol and 7β-hydroxycholesterol, Δ^4-cholestene-3β,6α-diol and Δ^4-cholestene-3β,6β-diol, afford further evidence of the relationship between mobility on paper partition chromatographic systems and structure of the steroid molecule as mentioned by Savard.[6] The greater mobilities of 7α-hydroxycholesterol and Δ^4-cholestene-3β,6β-diol over those of the 7β- and 6α-epimers, respectively, may be related to the conformation of the hydroxyl group of the sterol molecule. Thus the polar (more hindered) conformations of the 7α-hydroxyl and the 6β-hydroxyl confer greater mobility on the partition chromatograms as compared with the equatorial (less hindered) conformations of the 7β- and 6α-hydroxyls.

(6) K. Savard, *J. Biol. Chem.*, **202**, 457 (1953).

(7) Cholesteryl acetate resists autoxidation in aqueous colloidal suspension; *cf.* S. Bergström and O. Wintersteiner, *J. Biol. Chem.*, **145**, 327 (1942).

(8) R. Schueoheimer, *Z. physiol. Chem.*, **192**, 86 (1930); *J. Biol. Chem.*, **110**, 461 (1935).

(9) V. A. Petrow, *J. Chem. Soc.*, 1077 (1937).

(10) A. Windaus and H. Lüders, *Z. physiol. Chem.*, **109**, 183 (1920).

TABLE II

RELATIVE MOBILITY OF SEVERAL STEROLS AND DERIVATIVES

Sterol	Solvent system A (R_f)	Solvent system B (R_c)	Color behavior with:[a] Liebermann-Burchard[b]	Color behavior with:[a] $SbCl_3$
Cholesterol	0.93	1.00	V → Bl-G	V
Cholestan-3β-ol	.96	1.00	.....	G
Δ⁴-Cholesten-3-one	.93	1.00	.....	..
Δ⁵-Cholestene-3β,4β-diol	.75	0.51	P → Bl → G	..
Δ⁴-Cholestene-3β,6α-diol	.42	.13	Y → Pe → V → Bl -Gr (Pe → V → Bl)	..
Δ⁴-Cholestene-3β,6β-diol	.25	.18	Rs → Bl-G → G	..
Δ⁵-Cholestene-3β,7α-diol	.65	.17	Rs → V → Bl	Bl
Δ⁵-Cholestene-3β,7β-diol	.61	.11	Rs → V → Bl	Bl
Δ⁵-Cholesten-3β-ol-7-one	.97	.15	.....	Bl-G
Δ³,⁵-Cholestadien-7-one	.85	.09	.....	O
Cholestane-3β,5α,6β-triol	.19	.02	Br	..
Cholestane-3β,5α-diol-6-one	.13	.02	Br	..
Cholesterol dibromide	.88	.87	Br (V)	..
Cholesteryl acetate	..	1.5	V → Bl-G	V

[a] Bl = blue, Rs = rose, Br = brown, P = pink, V = violet, G = green, O = orange, Y = yellow, Pe = peach, Gr = gray. [b] Color behavior was the same for both chromatographic systems except as noted; in these cases the sequences in parentheses are for system A. The color sequences are characteristic and reproducible.

The presence of any of the several products associated with autoxidation of cholesterol in colloidal dispersion other than the 7-hydroxycholesterols could not be demonstrated in the debromination mixtures. Although cholestane-3β,5α,6β-triol was not detected, material of similar mobility but different color behavior was found (spot I in both systems). Neither Δ⁴-cholestene-3β,6β-diol nor Δ⁵-cholestene-3β,4β-diol was detected.[11] Examination of the sterol mixtures obtained by autoxidation of cholesterol by the procedure of Mosbach, *et al.*,[12] indicated the presence of cholestane-3β,5α,6β-triol both epimers of 7-hydroxycholesterol and cholesterol. The presence of 7-ketocholesterol could not be settled because of inadequate separations. Other products of autoxidation, Δ³,⁵-cholestadien-7-one and Δ⁴-cholesten-3-one,[12] could not be detected.

Acknowledgment.—The author is grateful for the gift of sterol samples from Dr. O. Wintersteiner, Squibb Institute for Medical Research; Dr. L. F. Fieser, Harvard University; Dr. F. Sondheimer, Syntex, S. A.; Dr. E. Schwenk, Worcester Foundation for Experimental Biology; and Dr. C. Kochakian, Oklahoma Medical Research Institute.

Experimental

Materials.—Cholesterol from commercial sources (Merck, USP) and from biosynthesis experiments with mammals was employed. The biosynthetic cholesterol was isolated from the unsaponifiable fractions of tissue by digitonin precipitation and regenerated with pyridine–ether. Samples examined varied in purity from crude low-melting material (110°) to preparations purified *via* the dibromide several times.

Cholesterol dibromide was prepared by the procedure of Schwenk[2a] from 20–100 mg. amounts of cholesterol and by Fieser's procedure[13] for larger quantities. Routinely the precipitated dibromide was debrominated directly following air-drying. Recrystallization from ethyl acetate–methanol gave material melting 112.0–115.5° (Kofler block). Recrystallization did not alter the results of these experiments.

Cholesterol dibromide was debrominated by a modification of the Windaus[14] procedure in which the dibromide was intimately mixed with zinc dust and stirred with acetic acid.[2a] Ether extracts prepared from the reaction mixture were washed with water, dilute sodium hydroxide solution, finally with water till neutral and evaporated under a nitrogen atmosphere. Debromination of cholesterol dibromide by use of the modified sodium acetate–ethanol,[9,10] sodium iodide–ethanol[8] and potassium acetate–ethanol[9] procedures was also investigated. In all cases extracts were reduced in volume under nitrogen.

Autoxidation products of cholesterol colloidally dispersed in aqueous media were prepared by the procedure of Mosbach, Nierenberg and Kendall.[12] These products were fractionated from cholesterol by partial precipitation with digitonin, partition between 85% aqueous methanol–pentane, and recrystallization from methanol. Sterol mixtures arising in the debromination reactions were also subjected to these fractionations.

Paper Chromatography.—The sterol mixtures obtained from the evaporated extracts (or from the partially concentrated fractions) were dissolved in chloroform to give a concentration of 5–10 μg./μl. Two systems of paper chromatography were used: system A, ascending technique, using Whatman No. 3MM filter paper sheets, developed with *o*-dichlorobenzene; and system B,[4] descending technique, using Whatman No. 1 and No. 3MM paper impregnated with β-phenoxyethyl alcohol (dipped into a 10% acetone solution of β-phenoxyethyl alcohol and momentarily dried in air) and developed with heptane (equilibrated with β-phenoxyethyl alcohol). The sterols were applied as spots of 5 mm. diameter, 5–50 μg. being used in system A, 50–100 μg. in system B. The papers were irrigated in sealed glass cylinders at 20°.

The sterols were located on the air-dried chromatograms by dipping into the Liebermann-Burchard reagent as prepared by Kingsley and Schaffert.[15] A nitrobenzene solution of antimony trichloride[16] was used for the sterols not giving the Liebermann–Burchard test. The spots were

(11) Δ⁴-Cholestene-3β,6β-diol is formed with cholesterol by the action of sodium acetate–alcohol on cholesterol dibromide; *cf.* (a) J. Lifschütz, *Z. physiol. Chem.*, **106**, 271 (1919); **111**, 253 (1920); (b) O. Rosenheim and W. W. Starling, *Chemistry & Industry*, **52**, 1056 (1933); *J. Chem. Soc.*, 377 (1937); V. A. Petrow, O. Rosenheim and W. W. Starling, *ibid.*, 677 (1938); reference 9; (c) A. Butenandt and E. Hausmann, *Ber.*, **70**, 1154 (1937). Cholesteryl acetate dibromide yields cholesterol and Δ⁵-cholestene-3β,4β-diol under the action of silver nitrate–pyridine, *cf.* reference 9.

(12) E. H. Mosbach, M. Nierenberg and F. E. Kendall, THIS JOURNAL, **75**, 2358 (1953); *cf.* O. Wintersteiner and S. Bergström, *J. Biol. Chem.*, **137**, 785 (1941); S. Bergström and O. Wintersteiner, *ibid.*, **141**, 597 (1941); **145**, 309 (1942).

(13) L. F. Fieser, THIS JOURNAL, **75**, 5421 (1953).

(14) A. Windaus, *Ber.*, **39**, 518 (1906).

(15) G. R. Kingsley and R. R. Schaffert, *J. Biol. Chem.*, **180**, 315 (1949).

(16) L. C. Clark, Jr., and H. Thompson, *Science*, **107**, 429 (1948); H. Rosenkrantz, *Arch. Biochem. Biophys.*, **44**, 1 (1953).

marked so soon as they appeared, and the distance from the point of application measured.

Conventional R_f values were determined from system A; however, the ratio of distance moved by a sterol to distance moved by cholesterol was employed for simplicity in system B, in that the solvent front was frequently obscured. This ratio, the R_c value, is converted to R_f by multiplying by the R_f of cholesterol (0.63) in the Neher–Wettstein system. The mobilities in Table I are averages of many determinations. Spots IIA and IIB (system B) did not separate in every case, and an average of 0.14 was determined for the unresolved spot. Chromatography of 25 μg. quantities for 5–6 hours usually resolved the mixture. The R_f for the 7-hydroxycholesterols determined in system A is a value for the mixture.

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Rauwolfia Alkaloids. II. Isolation and Characterization of Two New Alkaloids from *Rauwolfia serpentina* Benth

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RECEIVED FEBRUARY 1, 1954

Within the last year several authors have reported the isolation of new alkaloids from the Indian medicinal plant, *Rauwolfia serpentina* Benth.[1] In connection with our work on the structure of reserpine, the sedative principle of this plant, it was mentioned that chromatography of the oleoresin fraction yielded some new alkaloids in addition to reserpine.[2] The present note deals with these two new indole alkaloids. Both compounds are weak bases and isolated by chromatography of the oleoresin fraction on acid-washed alumina using benzene–chloroform mixtures as an eluant.

The first of the two alkaloids, tentatively called "alkaloid F," analyzed well for a $C_{21}H_{24}N_2O_3$ compound. This formulation was substantiated by the analysis of a hydrochloride. Comparison of the ultraviolet and infrared spectra (Fig. 2 and Fig. 1, A, B, respectively) of this alkaloid and tetrahydroalstonine (I)[3] indicates a close relationship of these two alkaloids. Their ultraviolet spectra are practically identical with the exception of a more intense shoulder at 248 mμ in the spectrum of tetrahydroalstonine. This difference was shown to be real since repeated recrystallizations of the sample did not alter the spectra.

The infrared spectrum of tetrahydroalstonine in chloroform differs in the following way from that of "alkaloid F": greater degree of hydrogen bonding of the NH group, broader and less symmetrical conjugated ester carbonyl band, 0.03 μ shorter wave length of the conjugated vinyl ether band and distinct differences in the longer wave length portion of the spectra. A series of spectra of both compounds at different concentrations in chloroform showed no evidence for intramolecular hydrogen bonding and no dependence of the breadth and shape of the carbonyl band upon concentration.

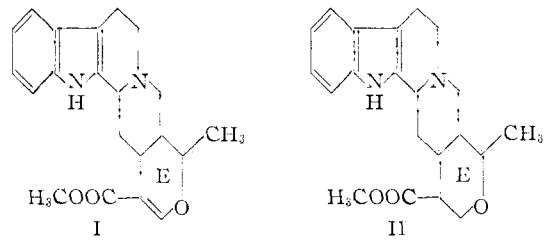
All of the observed differences between the spectra and the pK'_a values (see Experimental) of tetrahydroalstonine and "alkaloid F" could arise from differences in steric configuration of these two alkaloids. Comparison of several of the possible stereoisomers of tetrahydroalstonine, constructed with Fisher–Taylor–Hirschfelder models, shows considerable differences in the freedom of rotation of the carbomethoxy group and in the environment of the tertiary nitrogen.

The physical and analytical data of "alkaloid F" suggest the possibility of its identity with py-tetrahydroserpentine. This alkaloid was first prepared by Bader and Schwarz[4] by catalytic hydrogenation of serpentine in methanol at *p*H 10. The infrared and ultraviolet spectra were quite similar to those of our "alkaloid F." Lack of an authentic sample of py-tetrahydroserpentine did not permit the establishment of its identity with "alkaloid F." As soon as this can be done it is proposed to alter the formula of py-tetrahydroserpentine[4] (II) to I thus making it a steroisomer of tetrahydroalstonine.[5]

TABLE I

PHYSICAL PROPERTIES OF PY-TETRAHYDROSERPENTINE[4] AND "ALKALOID F"

Compound	M.p., °C.	$[\alpha]_D$	Infrared spectra, μ Ester CO	—C=C—O—	Solvent
Py-tetrahydroserpentine	249–250	−37 ± 6; −33 ± 6 (MeOH)	5.89	6.21	Methylene chloride
"Alkaloid F"	253–254	−44.6; −44.2 (MeOH–$CHCl_3$)	5.90	6.18	Chloroform
Py-tetrahydroserpentine HCl	276–277				
"Alkaloid F" HCl	264–265				



I

II

The second alkaloid, tentatively called "alkaloid A," analyzed well for a $C_{22}H_{26}N_2O_4$ compound. The formula was substantiated by analysis of a hydrochloride. The infrared and ultraviolet spectra of the alkaloid (Fig. 1 and Fig. 2, D) suggested a very close relationship to tetrahydroalstonine with a methoxy group at the 11-position of the heteroyohimbane ring system (III). Using the technique described in the previous paper,[2] we have recorded the composite infrared spectrum of tetrahydroalstonine and 2,3-dimethyl-6-methoxyindole at equimo-

(1) A. Stoll and A. Hofmann, *Helv. Chim. Acta*, **36**, 1143 (1953); K. Bodendorf and H. Eder, *Naturwissenschaften*, **40**, 342 (1953).

(2) N. Neuss, H. E. Boaz and J. W. Forbes, THIS JOURNAL, **75**, 4870 (1953); **76**, 2463 (1954).

(3) We thank Dr. R. C. Elderfield, of the University of Michigan, for the generous sample of tetrahydroalstonine. This structure for tetrahydroalstonine was first proposed by E. Schlittler, H. Schwarz and F. Bader, *Helv. Chim. Acta*, **35**, 271 (1952).

(4) F. Bader and H. Schwarz, *ibid.*, **35**, 1594 (1952).

(5) In a recent paper dealing with structure elucidation of tetrahydroalstonine, F. E. Bader, *ibid.*, **36**, 215 (1953), mentioned that there is a possibility that a double bond in the ring E of py-tetrahydroserpentine might have been overlooked.