TABLE I

Fraction	Methanol in solvent, %	Volume of solvent, ml.	Wt. solid eluted, mg.	Alkaloids present[a]
1	0	300	0	None
2	.5	250	50	Tetrahydroalstonine
3	.5	220	60	Tetrahydroalstonine + aricin
4	1.0	420	50	Ajmalicine
5	2.0	150	5	Unidentified alkaloid A
6	3.5	270	15	Reserpine
7	5	100		Reserpine
8	25	100	35	Reserpine
9	100	150	20	Unidentified alkaloid B

[a] As indicated by chromatography with benzene–cyclohexane solvent on formamide-impregnated paper.

anol to yield 20 mg. (0.002%) of reserpine, m.p. 265–266°, mixed m.p. with an authentic sample not depressed. The infrared spectrum and R_f values confirmed the identity of this material. A quantitative assay on fractions 7 and 8 indicated the presence of approximately 0.004% reserpine.

Ajmaline.—Fraction B from the original alkaloid separation was dissolved in a minimum of hot methanol, and held in the refrigerator overnight. Colorless crystals of ajmaline, 3.5 g., m.p. 148–152°, separated, and were removed by filtration. An additional 2.1 g. (total yield, 1.35%) of crystalline ajmaline were obtained on concentrating the mother liquors. A portion of this material was purified by recrystallization from aqueous methanol to yield pure ajmaline, m.p. 158–160° dec. The identity of the product was confirmed by comparison of the infrared spectrum and R_f values with those of an authentic sample.

The ajmaline mother liquors, 12 g. of dry weight, appeared from paper chromatography to contain in addition to substantial additional amounts of ajmaline, an unidentified weakly basic alkaloid, and a strong base similar in behavior to alstonine. However, alstonine was not isolated in pure form.

Acknowledgment.—We are indebted to Mrs. A. Paradies for her very capable assistance with this problem, and to Mr. W. H. Boegemann for the paper chromatographic studies. We should like to express our thanks to Dr. R. C. Elderfield for a generous sample of pure alstonine.

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The Structure of Frankincense Gum

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Frankincense gum from *Boswellia carterii* like myrrh gum[3,4] is an oleogum resin which contains some polysaccharide material. We report some properties of this carbohydrate portion. Owing to the small quantity of material available and because of large losses encountered in purifying it, this work is incomplete.

The gum was hydrolyzed with *N* sulfuric acid and the neutralized hydrolysate ($BaCO_3$) fractionated on cellulose in the usual way.[5]

D-Galactose and L-arabinose were thus isolated and identified. In addition two other substances, possibly fucose and rhamnose, were isolated, but in trace amounts only. The uronic acid was obtained as the barium salt and shown to be 4-*O*-methyl-D-glucuronic acid.

Although the sugars present in this gum are the same as those in gum myrrh[4] the molar ratios are quite different, *viz.*, galactose–arabinose–methylglucuronic acid, 7:1:4 for frankincense gum as compared with 8:2:7 for gum myrrh. The specific rotations are also quite different (−8 and +32°, respectively). On the other hand, their equivalents are of the same order of magnitude (545 and 460, respectively).

Autohydrolysis of these two gums was carried out under comparable conditions, and the changes in the composition of the hydrolysates followed by spotting samples on paper at intervals of time. Chromatograms in each case were run in both basic and acidic baths. Arabinose appeared in the hydrolysates at about the same time (6–9 hours) in each case, galactose rather sooner in frankincense (*ca.* 30 hours) than in myrrh (*ca.* 50 hours). Aldobiuronic acid also appeared sooner in the former (*ca.* 50 hours) than in the latter (*ca.* 70 hours). No other oligosaccharide materials were detected on the chromatograms.

Chromatography of an hydrolysate (6 hours with *N* sulfuric acid) in solvent (a) for 72 hours revealed a spot in the same position as that for 6-*O*-β-(4-*O*-methyl-D-glucuronosyl)-D-galactose. This acid together with 4-*O*-α-(4-*O*-methyl-D-glucuronosyl)-D-galactose occurs in gum myrrh.[4] The apparent lack of a 1,4-linked aldobiuronic acid in frankincense gum indicates a fundamental difference between two polysaccharides, which otherwise have several features in common.

Experimental

Unless otherwise stated, concentration of solutions was carried out at 40° (40 mm.) and specific rotations were measured in aqueous solution. Paper chromatograms were run in (a) ethyl acetate–acetic acid–formic acid–water (18:3:1:4)[6] or (b) butanol–pyridine–water (9:2:2).

Isolation and Purification of the Polysaccharide.—The gum resin (44 g.) in the form of small yellow nodules was extracted with hot acetone; evaporation of the extract left a pale yellow oil (17 g., 39%). The residue was dissolved in water (500 cc.), heated to about 85° and a slurry of cadmium hydroxide from cadmium sulfate (10.8 g.) and *N* sodium hydroxide (87 cc.) was added with rapid stirring. The mixture was centrifuged and the clear liquid shaken with a mixture of chloroform (0.25 vol.) and butanol (0.1 vol.) for 1 hour and then centrifuged.[7] The chloroform–protein gel formed a thin layer between the clear aqueous and chloroform layers. The aqueous layer was decanted and the process repeated until no further gel formed (4 treatments). The aqueous solution was then passed through columns of IR-120 and IRA 400 resins, and the gum acid (4.8 g.) was isolated as a white material by precipitating it in ethanol. It was collected on the centrifuge, washed and then dried at 60° (0.2 mm.), $[\alpha]^{19}D$ −8 ± 1° (*c* 1.0). Found: N, 0.5; OMe, 5.4; sulfated ash 0.37%, equiv. (by titration), 560 (545, on ash and protein free basis). Chromatography of the neutralized ($BaCO_3$) hydrolysate of this material (hydrolyzed for 16 hours in *N* sulfuric acid at 100°) gave spots (with *p*-anisidine hydrochloride) corresponding to galactose, arabinose and a monomethyl uronic acid. Extremely faint spots were present corresponding to fucose and rhamnose.

A quantitative determination of the non-acidic reducing sugars, after separation in the normal way on a chromato-

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gram, was done by the micro Somogyi method.[8] Galactose and arabinose were found in the approximate molar ratio 7:1.

Examination of the Non-acidic Reducing Sugars.—The gum acid (1.5 g.) was heated with N sulfuric acid (30 cc.) for 17 hours and then neutralized with IR-4B resin. The resin was filtered off and the solution concentrated to a sirup, which was chromatographed on a column of cellulose, using half-saturated aqueous butanol. The first fraction gave L-arabinose (0.07 g.), m.p. and mixed m.p. 154–157° after recrystallization from methanol–acetone, $[\alpha]^{20}D$ +109 ± 4° (c 0.6). The other fraction gave D-galactose (0.46 g.), m.p. and mixed m.p. 165–167° after recrystallization from methanol $[\alpha]^{20}D$ +83 ± 3° (c 1.7). Minute traces of two other materials which corresponded to fucose and rhamnose on the chromatogram were obtained, but they were not present in sufficient amount to be identified.

The Uronic Acid.—The gum acid (2.1 g.) in 2 N sulfuric acid (25 cc.) was heated on a boiling water-bath for 16 hours. The neutralized ($BaCO_3$) and filtered hydrolysate was poured into ethanol, and the precipitated barium salt collected (centrifuge). This salt (0.4 g.), after drying, was refluxed with 2% methanolic hydrogen chloride (10 cc.) for 6 hours after which it was neutralized (Ag_2CO_3), filtered and evaporated, leaving the methyl ester methyl glycoside as a sirup (0.4 g.). The glycoside was treated with methanolic ammonia and allowed to stand for several days. Evaporation yielded a sirup which crystallized on trituration with absolute ethanol. Recrystallization of this from ethanol gave the amide of methyl-4-*O*-methyl-α-D-glucuronoside, m.p. and mixed m.p. 232–235°, $[\alpha]^{25}D$ +150° (c 0.6).

Partial Hydrolysis.—The gum acid in N sulfuric acid was heated at 100° for 6 hours and the neutralized ($BaCO_3$) hydrolysate chromatographed in solvent (a) for 72 hours. An aldobiuronic acid spot with R_{Ga} (relative to galactose) 0.51 corresponded in position to the spot for 6-*O*-β-(4-*O*-methyl-D-glucuronosyl)-D-galactose from gum myrrh.[4] A spot corresponding to 4-*O*-α-(4-*O*-methyl-D-glucuronosyl)-D-galactose was not detected. There was a strong single spot of R_{Ga} 0.18, which might have been an aldotriuronic acid.

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Isolation and Characterization of 6,7-Dimethyl-9-(2′-hydroxyethyl)-isoalloxazine as a Bacterial Fermentation Product of Riboflavin

BY H. T. MILES AND E. R. STADTMAN

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Bacteria that catalyze the anaerobic decomposition of riboflavin have been obtained in a partially purified state by selective (serial) enrichment on a growth medium containing riboflavin as the major carbon source. During fermentation, the riboflavin, which is present in the growth medium mainly as an insoluble suspension, is converted to a less soluble green substance (1–3 days) which is subsequently converted into a red substance and ultimately (4–8 weeks) into an orange compound.

In order to characterize the latter compound, an experiment was set up as follows: 500 ml. of medium containing 5.0 g. of riboflavin, 500 mg. of yeast extract and mineral salts[1] were inoculated with 5.0 ml. of a partially purified enrichment culture of the riboflavin decomposing bacteria. The mixture was incubated for several weeks at 37° under strictly anaerobic conditions. During this time, the typical color changes described above took place, and most of the riboflavin disappeared. The orange precipitate which had accumulated was filtered and washed in turn with water, methanol and ether. The precipitate was dissolved in 4 N potassium hydroxide giving a dark green solution, which was centrifuged to remove bacterial cell debris.

Part of the green solution was treated with 10 N sulfuric acid to give a gelatinous green precipitate, which on filtering on a Büchner funnel overnight became orange. In order to obtain crystalline material, the remainder of the green, basic solution was shaken with ethyl acetate, slow hydrolysis of which led to a gradual acidification and resulted after several hours in the deposition of a crop of orange crystals. The compound had an absorption spectrum very similar to that of riboflavin (λ_{max} at 222, 268, 260 and 445) and had a yellow fluorescence in ultraviolet light, but could be readily separated from riboflavin by paper chromatography using as solvent system butanol–acetic acid–water (4:1:5, upper layer); the fermentation product had an R_f value of 0.45 and riboflavin 0.20.[2]

Treatment of the substance with periodic acid showed negligible periodate consumption by arsenite titration,[3] indicating that no adjacent hydroxyl groups were present.

The infrared spectrum showed a band at 3.11 μ, suggesting that at least one hydroxyl group was present.

The compound was acetylated with acetic anhydride and pyridine at 0°. A chloroform-soluble product was obtained which was separable from the parent compound by paper chromatography in the butanol–acetic acid–water or a butanol–pyridine–water solvent system (3:4:3). R_f values of the parent compound and of its acetate were 0.45 and 0.63, respectively, in the former solvent system and 0.66 and 0.83 in the latter.[2] The acetylated derivative lacked the 3.11 μ band in the infrared and had an additional ester carbonyl band at 5.75 μ; other bands were at 5.83, 5.98, 6.32 and 6.45. The infrared spectrum of the parent compound had bands at 5.78, 5.96, 6.32 and 6.45 μ.

The analyses indicated one hydroxyl group on a two-carbon side chain attached to the flavin ring. On the basis of the foregoing the most probable structure is 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine.

Anal.[4] Calcd. for $C_{14}H_{14}N_4O_3$: C, 58.73; H, 4.93; N, 19.57. Found: C, 58.25, 58.33; H, 4.58, 4.35; N, 20.31, 20.27. Calcd. for the acetate, $C_{16}H_{16}N_4O_4$: C, 58.53; H, 4.91; N, 17.07; Ac, 13.1. Found: C, 58.82, 58.29, 58.36; H, 4.72, 4.82, 5.09; N, 16.72, 16.42; Ac, 12.24, 12.34.

A sample of this compound, recently prepared from riboflavin, was obtained through the courtesy of Dr. H. G. Petering[5] and found to have an infrared spectrum superimposable with that of the bacterial fermentation product, confirming their identity. The spectra of the acetates were likewise superimposable.

Isolation of the Green Intermediate.—As mentioned above, during the fermentation of riboflavin, substances were observed having colors other than the characteristic yellow of flavins. Both red and green compounds were precipitated from the solution early in the fermentation.

The light green substance which accumulates as a transient intermediate was isolated by the following fermentation procedure: 5.0 g. of riboflavin was suspended in 25 ml. of water, and the suspension was sealed in a cellophane bag. The cellophane bag was then placed in one liter of medium containing mineral salts and yeast extract. The medium was inoculated with riboflavin decomposing bacteria and was incubated at 37° for several weeks. Under these conditions, the riboflavin suspension is physically separated from the rest of the bacterial culture. As dissolved riboflavin diffused through the cellophane membrane in to the surrounding medium, it was converted by the bacteria to the green intermediate, which crystallized out. The cellophane membrane thus served to maintain separation of the insed

(1) One hundred ml. of growth medium contained KH_2PO_4, 0.65 g.; K_2HPO_4, 0.175 g.; $MgSO_4 \cdot 7H_2O$, 20 mg.; $CaSO_4 \cdot 2H_2O$, 1 mg.; $FeSO_4 \cdot 7H_2O$, 0.5 mg.; $MnSO_4 \cdot 4H_2O$, 0.25 mg.; $NaMoO_4 \cdot 2H_2O$, 0.25 mg.; and $Na_2S \cdot 9H_2O$, 30 mg.

(2) The numerical values varied slightly in different runs.

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(4) The microanalyses were performed by Clark Microanalytical Laboratory of Urbana, Illinois.

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