infrared spectrum shows peaks at 1765, 1230, and 1065 cm.$^{-1}$.

Dihydroamaryllisine was prepared by stirring an ethanol solution of 17 mg. of the alkaloid with 15 mg. of 10% Pd–C under an atmosphere of hydrogen. The solution was filtered and the solvents were removed under reduced pressure. The residue was sublimed to provide material, m.p. 243–245°, unchanged by crystallization from ethyl acetate; $[\alpha]^{24}D$ −11°, $[\alpha]^{24}_{436}$ −22° (*c* 0.68, ethanol).

Acknowledgment.—We wish to thank Professor K. Biemann in whose laboratory preliminary mass spectrometric studies were carried out by A. L. B.

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN CO., KALAMAZOO, MICHIGAN]

Celesticetin. III. The Partial Structure[1-3]

BY HERMAN HOEKSEMA AND JACK W. HINMAN

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The reactions and determinations leading to the partial structure of celesticetin are presented.

Celesticetin is an antibiotic which has activity against Gram-positive organisms both *in vitro* and *in vivo*.[1] It is readily extracted from fermentation broths by organic solvents under neutral conditions.[2] The compound is most conveniently isolated as the crystalline oxalate or salicylate salts. These are reconverted with ease to an amorphous free base which has been shown to be 96% pure by countercurrent distribution. The compound has the molecular formula[4] $C_{24}H_{36}N_2O_9S$ and contains one C-methyl, one N-methyl, and one methoxyl group. Potentiometric titrations in water and dimethylformamide reveal a basic function of pK_a' 7.7, and an acidic one of pK_a' 9.8, with an equivalent weight per group of 517 ± 20.

Structural studies show celesticetin to have the partial structure **1**. This is established by a study of celesticetin derivatives, the products of acidic and basic hydrolyses, and nickel desulfurization.

Celesticetin base displays characteristic salicylate ester type absorption in the ultraviolet with maxima in 0.01 *N* alcoholic potassium hydroxide at 248 mμ, $E^{1\%}_{1\,cm}$ 130.3, and at 341 mμ, $E^{1\%}_{1\,cm}$ 103.7. In 0.01 *N* ethanolic sulfuric acid the maxima shift to 240 mμ, $E^{1\%}_{1\,cm}$ 183.7, and to 310 mμ, $E^{1\%}_{1\,cm}$ 80.6. In chloroform solution, infrared absorptions at 3300 cm.$^{-1}$ characteristic of hydroxyl and imino functions, at 1670 cm.$^{-1}$ originating, as will appear below, in a salicylic ester grouping, and at 1645 and 1517 cm.$^{-1}$ suggesting a monosubstituted amide, can be distinguished. Positive ferric chloride and Molisch tests, and negative Benedict, ninhydrin, and iodoform reactions are observed. Precipitates may be obtained with bromine water, Millon's reagent, and mercuric chloride. In aqueous solution, celesticetin consumes 4 moles of periodate rapidly, and part of an additional one slowly. Of these, two would be expected to oxidize sulfur to the sulfone.

Acetylation of celesticetin affords a tetraacetate, **2**, $C_{32}H_{44}N_2O_{13}S\cdot HCl$, crystalline as the hydrochloride. The only titrable group remaining in **2** is a basic function, pK_a' 7.7.

The presence of a salicylate ester is confirmed chemically by alkaline hydrolysis in 1 *N* sodium hydroxide. This provides an acid-insoluble fraction, identified as salicylic acid. The phenolic hydroxyl accounts for the weakly acidic function of celesticetin. From the filtrate, evaporated to dryness at pH 8.3, an alcohol-soluble material which fails to crystallize may be obtained. Countercurrent distribution in 1-butanol, water, and ammonia shows this amorphous substance to be 96% pure. This compound, desalicetin, **3**, retains the N-methyl, O-methyl, and C-methyl groups and has the molecular formula $C_{17}H_{32}N_2O_7S$. It is a base, pK_a' 7.8, which is optically active ($[\alpha]D$ +175° (*c* 1, ethanol)) and shows only end absorption in the ultraviolet. The ester and aromatic bands in the infrared have disappeared, but those attributed to the monosubstituted amide, now shifted slightly to 1650 and 1525 cm.$^{-1}$, remain. Desalicetin is cleaved by 4 moles of periodate rapidly and part of an additional mole slowly. Following removal of excess periodate and acidic hydrolysis, treatment with 2,4-dinitrophenylhydrazine permits isolation of glyoxal 2,4-dinitrophenylosazone. Desalicetin also gives a tetraacetate upon acylation with acetic anhydride. This can be crystallized as the hydrochloride, $C_{25}H_{40}N_2O_{11}S\cdot HCl$ (**4**). Although desalicetin has low *in vitro* antibacterial activity, it is interesting to note that it is fully as active *in vivo* on a molecular basis as is celesticetin.

Desulfurization of celesticetin with Raney nickel, which has been washed with a citric acid buffer at pH 3 to minimize alkaline hydrolysis, affords ethyl salicylate as an ether-soluble fragment. This shows that the salicylic acid moiety is bound through an ester linkage to a two-carbon fragment. One of these two carbons must then be linked to the sulfur atom.

The water-soluble product of desulfurization is isolated by evaporation at pH 8, and extraction of the residue with ethanol, then conversion to the hydrochloride, $C_{13}H_{28}N_2O_6\cdot HCl$. This is called 1,5-anhydrocelesticetitol, **5**. This optically active, nonreducing material can also be obtained by desulfurization of desalicetin. It has one basic function, pK_a' 7.8. Only 2 moles of periodate are consumed rapidly by 5, again with slow additional uptake; 1 mole of formic acid is liberated. The desulfurized product contains three acylable hydroxyls, which can be acetylated to form a crystalline triacetate hydrochloride, $C_{21}H_{34}N_2O_9\cdot HCl$, **6**. In view of the periodate data these hydroxyls must be adjacent. Since the fourth oxygen in **5** is in the methoxyl function, and the fifth in the

(1) C. DeBoer, A. Dietz, J. R. Wilkins, C. N. Lewis, and G. M. Savage, "Antibiotics Annual (1954–5)," Medical Encyclopedia, New York, N. Y., p. 831.

(2) H. Hoeksema, G. F. Crum, W. H. Devries, *ibid.*, (1954–5), p. 837.

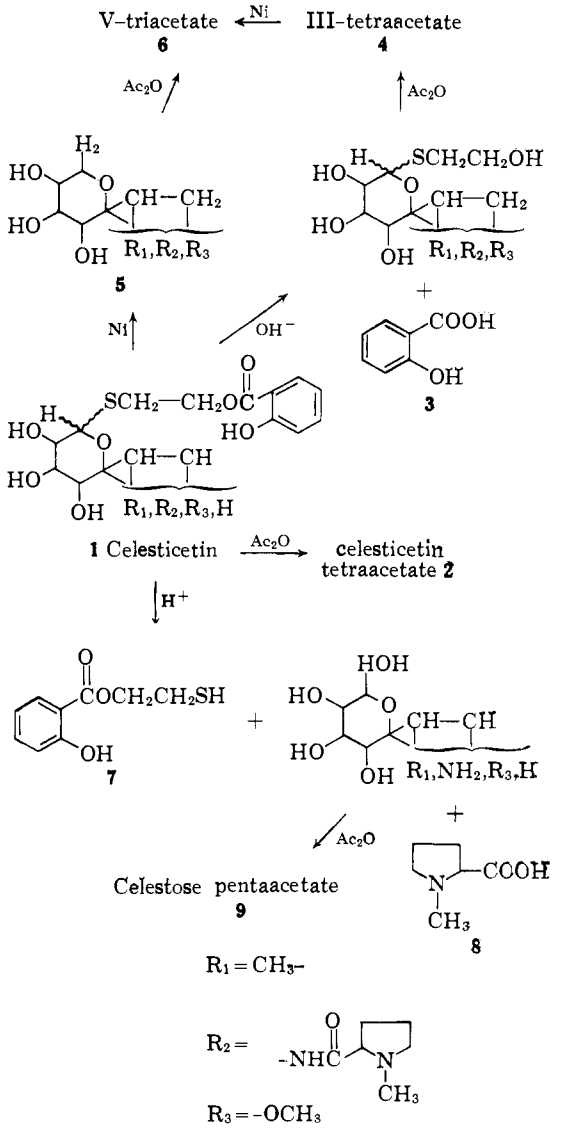
(3) J. W. Hinman and H. Hoeksema, presented in part at the 126th National Meeting of the American Chemical Society, Dallas, Texas, April, 1956.

(4) The analytical data actually require a range of H_{36-40}.

carbonyl, only one is unaccounted for. In view of the three sites of unsaturation, and the carbohydrate-like nature of celesticetin, it is highly probable that the remaining oxygen is present as a cyclic ether in **5** (and hemiacetal in **1** and **3**). The periodate uptake requires a pyranose ring rather than a furanose.

Vigorous acid hydrolysis of celesticetin, using 6 to 12 *N* hydrochloric acid with heating, decomposes the sugar moiety, but permits the isolation of two products. The first of these involves the salicylic acid portion. It is isolated by steam distillation as the hydrolysis progresses. The second is an amino acid, isolated from the aqueous residue after steam distillation has removed the first substance.

The steam-distilled product may be further purified by distillation at about 110° at 50 μ. This compound, $C_9H_{10}O_3S$, **7**, is optically inactive, contains no



C-methyl groups, and gives positive nitroprusside and ferric chloride tests. Infrared absorptions at 3190, 2560, 1674, 1616, 1589, 1487, 756, and 697 cm.$^{-1}$ suggest hydroxyl and aromatic ester functions. The ultraviolet spectrum, with maxima at 238.5 and 308 mμ in acidic ethanol, is characteristic of salicylates. These data then are consistent for β-thioethyl salicylate.

The analyses of the crystalline amino acid hydrochloride are somewhat inaccurate, owing to the nature of the compound, but they are indicative of the formula $C_6H_{11}NO_2 \cdot HCl$, **8**. Group analyses show one N-methyl group to be present, and potentiometric titrations indicate a compound with an equivalent weight of 165.5, with an acid group, pK_a' 1.8, and a base, pK_a' 10.4. The data are consistent for N-methylproline, commonly called hygric acid. More positive identification is made by conversion to the cupric salt, which is identical with that from L-hygric acid synthesized from L-proline.[5] This moiety then accounts for the basic group in celesticetin and also for the slow additional uptake of up to 1 mole of periodate, since proline amide and hygric acid amide behave in a similar fashion. The moiety is postulated to arise from the cleavage of the amide bond, giving rise to the new acid function.

A milder hydrolysis, heating with 4 *N* sulfuric acid, affords the carbohydrate residue, celestose, which can be crystallized as the pentaacetate, $C_{19}H_{29}NO_{11}$, **9**. Celestose is a reducing sugar containing one acylable amino nitrogen. The latter would be expected to arise from the basic part of the monosubstituted amide in the hydrolysis.

The data which have been presented appear to be consistent with the formulas and transformations illustrated in the accompanying chart. Additional evidence and assignment of the remaining groups will be recorded in a later publication.

Experimental

Isolation of Celesticetin. Celesticetin Salts.—A 260-l. quantity of a *Streptomyces caelestis* fermentation broth, containing 28 μg./ml. of celesticetin, was filtered through Supercel. Following an adjustment with dilute sulfuric acid to pH 7.8, the filtrate was extracted with 50 l. of methylene chloride. The solvent was evaporated at low pressure to leave 150 ml. of a tacky residue which was hardened by trituration with 750 ml. of hexane. After drying, this precipitate weighed 6.1 g. and assayed 750 μg./mg. of celesticetin by a *B. subtilis* agar plate assay using a neomycin standard. The recovery from the beer was 64% of the antibacterial activity.

Celesticetin Oxalate.—Oxalic acid dihydrate (0.32 mmole) was added to crude celesticetin (200 mg., est. 0.3 mmole) in 20 ml. of methanol. This solution was warmed, then added with stirring to 125 ml. of anhydrous ether. Ether trituration of the resulting gum afforded 120 ml. of white powder which was recrystallized from methanol–ether to yield 70 mg. of white needles melting 147–152°. Its specific rotation was $[\alpha]^{24}D$ +105.8 (*c* 0.5%, 1 *N* hydrochloric acid). Solubility analysis showed a purity of 99% for celesticetin oxalate.

Anal. Calcd. for $C_{26}H_{40}N_2O_{13}S$: C, 50.31; H, 6.45; N, 4.51; S, 5.17. Found: C, 50.72; H, 6.20; N, 4.75; S, 5.22.

Celesticetin Salicylate.—Salicylic acid (3.0 g., 21.7 mmoles) was added to 9.0 g. (est. 11.5 mmoles) of crude celesticetin base in 50 ml. of methanol. Evaporation of the solvent left a gummy residue which was crystallized and recrystallized from ethyl acetate to yield 4.9 g. of monoclinic crystals melting at 139°. The specific rotation for this compound was $[\alpha]^{24}D$ +99.0° (*c* 0.5%, 1 *N* hydrochloric acid).

Anal. Calcd. for $C_{31}H_{44}N_2O_{12}S$: C, 55.67; H, 6.62; N, 4.19; S, 4.79. Found: C, 55.70; H, 6.13; N, 4.21; S, 4.92.

Celesticetin Base (1).—Celesticetin oxalate (20.0 g.) was dissolved in 200 ml. of water and to this was added 100 ml. of methylene chloride. The mixture was stirred very vigorously during adjustment of the pH to 7.5 with 20% sodium hydroxide. The methylene chloride layer was separated and washed twice with 25 ml. of water, then dried over sodium sulfate, and evaporated to dryness at reduced pressure to yield 10.4 g. of a glassy foam. This foam was analyzed by a countercurrent distribution using the system 1-butanol–water–acetic acid (4:5:1). After 200 transfers a single peak was observed with a *K*-value of 0.77 (Fig.

(5) E. Schulze and G. Trier, *J. Physiol. Chem.*, **67**, 324 (1910).

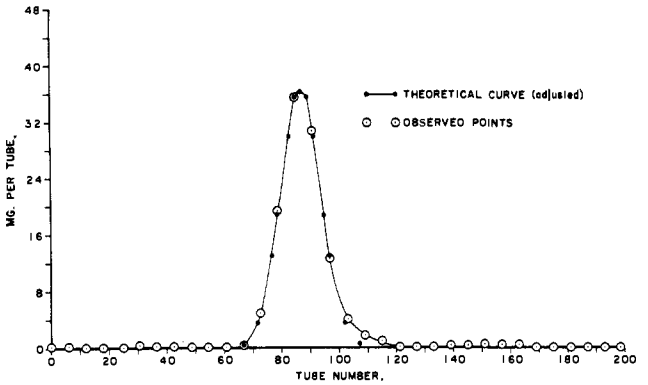


Fig. 1.—Countercurrent distribution of celesticetin.

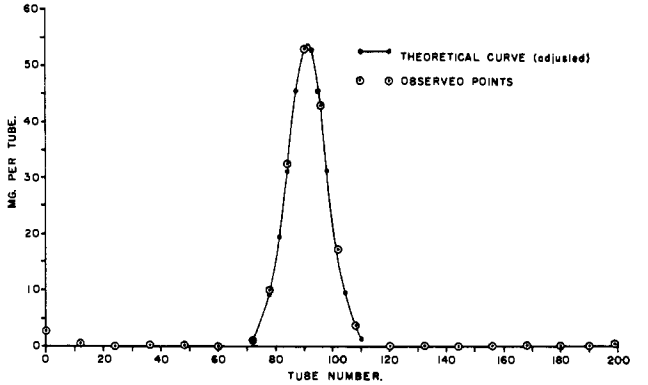


Fig. 2.—Countercurrent distribution of desalicetin.

1). The specific rotation was found to be $[\alpha]^{24}D$ +124.0° (*c* 0.5% in 1 *N* hydrochloric acid.

Anal. Calcd. for $C_{24}H_{38}N_2O_9S$: C, 54.58; H, 7.25; N, 5.28; S, 6.04. Found: C, 54.87; H, 6.75; N, 5.30; S, 6.02.

Celesticetin Tetraacetate (2).—A solution containing 2.0 g. (3.83 mmoles) of celesticetin base, 5 ml. (excess) of acetic anhydride, and 25 ml. of pyridine was stored at 25° for 16 hr., then heated for 3 hr. under reflux. After cooling, it was diluted with water, neutralized with solid sodium bicarbonate, and evaporated to dryness under reduced pressure. The residue was dissolved in the system water (100 ml.) and ether (50 ml.). The water layer was subsequently extracted two more times with 50-ml. portions of ether. The combined ether layers were washed with water and dried over magnesium sulfate. The solvent was evaporated *in vacuo* to leave 2.36 g. (3.44 mmoles, 90% yield) of a glassy residue.

Anal. Calcd. for $C_{32}H_{46}N_2O_{13}S$: C, 55.00; H, 6.64; N, 4.01; acetyl, 24.2. Found: C, 54.88; H, 6.42; N, 4.00; acetyl, 24.5.

Celesticetin Tetraacetate Hydrochloride.—Treatment of 540 mg. (0.79 mmoles) of **2** in 20 ml. of ether with dry hydrogen chloride yielded a white precipitate which crystallized from ethyl acetate (350 mg., 0.48 mmoles) in 60% yield. After a recrystallization from chloroform, ethyl acetate, and ether, these crystals melted at 180–185°.

Anal. Calcd. for $C_{32}H_{47}ClN_2O_{13}S$: C, 52.27; H, 6.44; N, 3.81; S, 4.36; acetyl, 22.8. Found: C, 52.55, 52.91; H, 6.42, 6.19; N, 3.88, 4.02; S, 4.51; acetyl, 21.96.

Base Hydrolysis: Desalicetin (3) and Salicylic Acid.—Celesticetin (10.6 g., 0.020 mole) was dissolved in 100 ml. of 1 *N* sodium hydroxide and kept at room temperature for 16 hr. After acidification to pH 1.5 with 2 *N* hydrochloric acid and cooling, a crystalline precipitate appeared. This was removed and identified as salicylic acid. Additional salicylic acid was removed by extraction for a total of 2.7 g. (98% of 1 mole). The aqueous solution, which now gave a negative ferric chloride test, was made alkaline to pH 8.3 with sodium hydroxide. This clear solution was evaporated to dryness *in vacuo* leaving a light colored residue. Extraction of the residue with hot absolute ethanol, followed by evaporation of the solvent, left a brittle, colorless glass which weighed 8.12 g.

A sample of this material was distributed countercurrently for 199 transfers in a system 1-butanol–water–concentrated ammonium hydroxide (10:9:1, v./v.). Solid analysis of this distribution permitted the tracing of a nearly theoretical curve (Fig. 2) and indicated a minimum 94–95% purity. Sodium chloride and water were found to be trace impurities.

Broth dilution tests showed desalicetin to inhibit *Streptococcus hemolyticus* L-2, *Diplococcus pneumoniae* F.I., *Streptococcus viridans* 25–11, and *Micrococcus pyogenes* var. *aureus* M-L (A + T + C) at 25 mcg./ml. *Micrococcus pyogenes* var. *aureus* (FDA —209) was inhibited at 12.5 mcg./ml. Desalicetin showed only end absorption in the ultraviolet. It was optically active, $[\alpha]^{25}D$ +175° (*c* 1.06%, ethanol).

Anal. Calcd. for $C_{17}H_{33}N_2O_7S \cdot H_2O$: C, 47.76; H, 8.25; N, 6.55; S, 7.50. Found: C, 47.85, 47.92; H, 7.98, 7.43; N, 6.52; S, 7.33.

Desalicetin Tetraacetate Hydrochloride (4).—Using the procedure described above for celesticetin, 2.0 g. of desalicetin was acetylated to yield 2.57 g. of a pale yellow, water-insoluble gum. Since this gum defined all attempts at crystallization, a solution in 150 ml. of anhydrous ether was treated with dry hydrogen chloride until no further precipitate appeared. The crude salt was recrystallized from chloroform and ether to yield 2.5 g. of product. The analytical sample (m.p. 201–202°) was also recrystallized from ethanol and ethyl acetate.

Anal. Calcd. for $C_{28}H_{40}N_2O_{11}S \cdot HCl$: C, 48.96; H, 6.74; N, 4.57; acetyl, 27.4. Found: C, 49.36, 49.50; H, 6.68, 6.72; N, 5.16, 5.12; acetyl, 26.6.

Desulfurization of Celesticetin: 1,5-Anhydrocelesticetitol (5) and Ethyl Salicylate.—W-6 Raney nickel (100 g.) was washed by slurrying twice with 200 ml. of 1.0 *M* citrate buffer, then once with 100 ml. of water. To a mixture of this catalyst in 400 ml. of water, vigorously stirred and heated to boiling, 100 ml. of aqueous solution containing 15 g. (0.023 moles) of celesticetin salicylate was added. After heating under reflux 10 min., the mixture was distilled until no oil could be detected in the 100 ml. of distillate. An ether (50 ml.) extract of this distillate was washed twice with 25 ml. of 5% potassium bicarbonate solution and once with water, then evaporated to 1 g. (28%) of colorless oil, which, after distillation *in vacuo*, yielded 300 mg. of oil. Identity with ethyl salicylate was confirmed by infrared and ultraviolet spectra and analyses.

The reaction-pot contents from the distillation were filtered and the nickel washed with hot acetone. The combined filtrates were concentrated, then freeze-dried to yield 7.6 g. This residue was dissolved in 75 ml. of water, acidified to pH 2 with hydrochloric acid, extracted twice with 50 ml. of ether to remove 2.5 g. of salicylic acid, then brought to pH 9 with sodium hydroxide. This solution, to which 1-butanol was added, was next azeotropically distilled to a butanol concentrate. It was then acidified with hydrogen chloride gas and filtered to remove the sodium chloride. When six volumes of ether was added to the solution, 4.32 g. of hygroscopic solid was precipitated. This was dissolved in water, treated with hydrogen sulfide to remove traces of nickel, then recovered by evaporation and again reprecipitated from butanol–ether. Potentiometric titration showed a single basic function, pK_a' 8.0 in water and an equivalent weight of 362 g. (calcd. 371.89 g.). *Anal.* Calcd. for $C_{15}H_{28}N_2O_6 \cdot HCl$: C, 48.84; H, 7.92; N, 7.60; Cl, 9.61. Found: C, 48.98; H, 8.39; N, 6.73; Cl, 9.30.

1,5-Anhydrocelesticetitol Triacetate (6) from Desalicetin Tetraacetate Hydrochloride.—A mixture of 1.5 g. of crystalline desalicetin tetraacetate hydrochloride, 15 g. of W-6 Raney nickel washed as before, and 80 ml. of 95% ethanol was heated under reflux with stirring for 2 hr. The catalyst was removed by filtration and washed thoroughly with hot 95% ethanol. The combined filtrates were concentrated *in vacuo* to 10 ml. and diluted to 20 ml. with water. This was adjusted to pH 8.5 and extracted with three 50-ml. portions of ether. The extract was dried over magnesium sulfate and evaporated to 0.75 g. (67%) of a colorless gum.

Anal. Calcd. for $C_{21}H_{34}N_2O_9$: C, 55.00; H, 7.47; N, 6.11; acetyl, 27.6. Found: C, 54.69; H, 7.15; N, 5.93; acetyl, 26.7.

This triacetate was converted to the hydrochloride by treatment of 300 mg. in 30 ml. of ether–Skellysolve B (50:50, v./v.) with hydrogen chloride. An 86% yield (280 mg.) of white hydrochloride was collected. Crystallization from chloroform and ether provided an optically active ($[\alpha]D$ +42.0° [*c* 1.0, water]) salt melting at 224–226°. Potentiometric titration in water revealed a single basic group of pK_a' 8.0, equiv., wt. 498 (calcd. 494.97). This compound consumed a trace, 0.23 mole, of periodate in 2 hr.

Anal. Calcd. for $C_{21}H_{34}N_2O_9 \cdot HCl$: C, 50.95; H, 7.13; N,

5.66; acetyl, 26.0. Found: C, 50.85; H, 7.48; N, 5.65; acetyl, 23.2.

Vigorous Acid Hydrolysis: β-Thioethyl Salicylate (7) and Hygric Acid (8).—Celesticetin salicylate (15 g., 0.022 mole) was added to 250 ml. of constant boiling hydrochloric acid which was being heated under reflux. After 5 min. of continued boiling, the mixture was chilled rapidly, diluted with 200 ml. of water, and extracted with 0.5 volume of ether. The boiling and extraction procedure was repeated twice. The combined extracts were shaken with 200 ml. of water containing 6 g. of mercuric chloride. The resulting white precipitate, when washed and dried, weighed 4.35 g. This was dissolved in 80 ml. of purified dioxane and treated with hydrogen sulfide. The 2.1 g. of precipitated mercuric sulfide was removed and the filtrate diluted with water, then extracted with 125 ml. of ether. After several water washings and drying over magnesium sulfate, the ether was evaporated, leaving an oily residue. This was distilled and a fraction boiling *ca.* 110°, 50 μ, was collected (1.1 g.).

Anal. Calcd. for $C_9H_{10}O_3S$: C, 54.53; H, 5.09; S, 16.17; CH_3–C, 0. Found: C, 55.09; H, 5.20; S, 16.77; CH_3–C, 0.

The aqueous residue from the initial extraction above was evaporated *in vacuo* to dryness. A 4-g. aliquot of the residue was dissolved in 40 ml. of water and washed with two 20-ml. portions of chloroform, then diluted to 160 ml. with water and washed with two 40-ml. portions of butanol. The water was next removed by evaporation *in vacuo* and the residue was dissolved in 40 ml. of ethanol and diluted with 200 ml. of ether. After the first gummy precipitate was discarded, 160 ml. of slower-growing white crystals were recovered. The 90 mg. of recrystallized product melted at 183–184°, had an acidic function (pK_a' 1.8) and a basic one (pK_a' 10.4 in water), and was optically active, $[\alpha]_D$ −70° (*c* 1%, water) lit. −81°. The equivalent weight was 165.5 (calcd. 165.6). Analyses for this compound were rather unsatisfactory, owing perhaps to loss of hydrogen chloride in drying.

Anal. Calcd. for $C_6H_{12}ClNO_2$: C, 43.50; H, 7.30; N, 8.46; Cl, 21.40; N-methyl, 17.6. Found: C, 44.40 to 45.50 (4 analyses); H, 7.02; N, 8.52, 9.63; Cl, 20.95; N-methyl, 6.4.

This material was converted to its copper salt by evaporation to dryness with copper hydroxide. The blue salt, then crystallized from chloroform, melted 208° dec. (lit. 200–208° dec.). Its infrared spectrum was identical with that of L-N-methylproline which was synthesized from L-proline *via* its betaine and methyl hydrate.

Milder Acid Hydrolysis of Celesticetin: Celestose Pentacetate (9).—A solution of 5 g. (0.0094 mole) of celesticetin in 100 ml. of 4 *N* sulfuric acid was heated under reflux for 15 hr. At this time a Van Slyke amino-nitrogen determination indicated 80% hydrolysis. The reaction mixture was cooled and filtered. The filtrate was extracted twice with 100 ml. of ether, then adjusted to pH 4.3 with barium hydroxide. The barium sulfate was removed and the filtrate was concentrated *in vacuo* to 70 ml. A 20-ml. aliquot of this was evaporated to dryness (*in vacuo*) and the residue was heated under reflux for 3 hr. with 25 ml. of pyridine and 5 ml. of acetic anhydride. After this solution was treated with water and neutralized with sodium bicarbonate, it was evaporated *in vacuo* to dryness. The residue was suspended in water and extracted with 50-ml. portions of ether. The pooled extracts, dehydrated over magnesium sulfate, were evaporated to dryness, yielding 0.39 g. of dark gum. From this, using ether and Skellysolve B, 100 mg. of tan crystals was obtained. A solution of these in ethanol, after treatment with carbon to decolorize, yielded 28 mg. of colorless needles after addition of ether and Skellysolve B. These exhibited a transition point at 215–216° and melted at 234–234.5°.

Anal. Calcd. for $C_{19}H_{29}NO_{11}$: C, 51.00; H, 6.53; N, 3.13; acetyl, 48.3; methoxyl, 6.9. Found: C, 51.26; H, 6.45; N, 3.06; acetyl, 48.7; methoxyl, 6.1.

Acknowledgment.—We wish to thank Mr. E. L. Caron for work on countercurrent distributions, and Dr. W. A. Struck and associates for analytical data.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY, LAFAYETTE, INDIANA]

The Synthesis of Polynucleotide–Celluloses and Their Use in the Fractionation of Polynucleotides[1]

BY P. T. GILHAM

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A method for the incorporation of chemically synthesized polynucleotides onto cellulose has been developed. By the polymerization of the appropriate mononucleotides, celluloses have been obtained to which thymidine, deoxyadenosine, or deoxycytidine polymers are attached at one of their ends by covalent linkages. Series of oligonucleotides can be bound to columns of these substituted celluloses in base-pairing complexes of different stabilities and subsequently can be fractionally eluted by using a temperature-gradient technique. The application of this method to separation of complex polynucleotides and to the sequence analysis of nucleic acids is discussed.

One of the approaches to the analysis of the fine structure of nucleic acids involves their specific degradation to smaller polynucleotides, the separation of these fragments, and the determination of their individual base sequences. Some success has been achieved in specific degradation by the use of enzymes and by the use of chemically modified nucleic acids while the fractionation of polynucleotides with ion-exchange resins[2] and DEAE–cellulose,[3] together with chromatography and electrophoresis,[4] has permitted the isolation of components up to the tetranucleotide level. However, the separation of mixtures of larger polynucleotides is expected to prove a difficult problem especially in cases where the mixtures contain polynucleotides of approximately the same size with approximately the same base ratio content. In the case of larger polynucleotides it is obvious that the distinctive property of their base sequences must be exploited in any method aimed at their separation and purification. We have briefly described[5] such a method, one that involves the use of celluloses to which chemically synthesized polynucleotides have been permanently attached. A mixture of polynucleotides can then be fractionated on these celluloses according to the ability of the individual components to form base-pairing complexes at various temperatures with the incorporated polynucleotides. This approach, involving base-pairing complexes, has also been used for the fractionation of nucleic acids by Bautz and Hall[6] and for the fractionation of large homologous ribo-

(1) This work has been supported by the National Institutes of Health, U. S. Public Health Service, and the National Science Foundation.

(2) E. Volkin and W. E. Cohn, *J. Biol. Chem.*, **205**, 765 (1953).

(3) M. Staehelin, *Biochim. Biophys. Acta*, **49**, 11 (1961).

(4) G. W. Rushizky and H. A. Sober, *J. Biol. Chem.*, **237**, 2883 (1962).

(5) P. T. Gilham, *J. Am. Chem. Soc.*, **84**, 1311 (1962).

(6) E. F. K. Bautz and B. D. Hall, *Proc. Natl. Acad. Sci. U. S.*, **48**, 400 (1962).