of the *p*H to 6.3 caused precipitation. After cooling overnight at −7° the solid was centrifuged off, washed with 10 ml. of cold 80% ethanol, and dried in a vacuum over phosphorus pentoxide. A yield of 226 mg. of white solid (CS-51RH) was obtained. A 1% solution of CS-51RH gave the following color tests: pinkish biuret, deep purple ninhydrin, strongly positive Millon test, very faint Molisch test and no reduction of Benedict reagent.

CS-56H Obtained by Acid Treatment of CS-56.—A solution of 44.3 g. of CS-56 in 600 ml. of 0.100 *N* sulfuric acid was refluxed for four hours. The protein component was isolated as described for CS-51RH. A yield of 22.4 g. was obtained. After two more reprecipitations involving carbon decolorization, a final yield of 17.0 g. of white solid, CS-56H, was obtained. Results of color tests on a 1% solution of CS-56H were: pinkish biuret, deep purple ninhydrin, positive Millon, positive Molisch, no reduction of Benedict reagent.

By evaporation of the alcoholic solution remaining after the first precipitation of CS-56H a yield of 10.7 g. of a brown hygroscopic powder containing 2.9% nitrogen was obtained. This is 26.8% of the original CS-56. A 1% solution of this carbohydrate mixture gave strongly positive Bial and aniline hydrochloride tests indicating its pentose nature. It contained 61% reducing sugar calculated as glucose. It yielded an unstable phenylosazone which melted constantly at 154–156° after several recrystallizations from 30% ethanol.

Fraction CS-56R.—To a solution of 4.5 g. of CS-56 in 250 ml. of water was added 100 ml. of a saturated solution of picric acid (excess). After standing overnight the suspension was centrifuged for one hour in the batch bowl of the Sharples supercentrifuge at 45,000 r. p. m. Ordinary centrifuging would not separate the stable picrate suspension. The gummy picrate was dissolved in 175 ml. of 0.05 *N* sodium hydroxide and the protein recovered by the method described for the cathodic picrates. A yield of 1.13 g. of white solid CS-56R was obtained. A 1% solution of CS-56R gave no reduction of Benedict reagent even after boiling for one hour with 3 *N* hydrochloric acid.

Fraction CS-56S.—The supernatant solution obtained by centrifuging the picrate of CS-56 (above) was poured into 1400 ml. of cold ethanol and cooled at 3° overnight. The solid was separated by centrifuging and washed with 20 ml. of cold ethanol. A yield of 1.27 g. of white solid (CS-56S) was obtained after drying in a vacuum over phosphorus pentoxide. A 1% solution of CS-56S did not reduce Benedict reagent, but after boiling one minute with 3 *N* acid the neutralized solution gave strong reduction.

CS-56RH.—A solution containing 500 mg. of CS-56R in 10 ml. of 0.1 *N* sulfuric acid was refluxed for four hours. The solid was isolated as described for CS-51RH. A yield of 167 mg. of CS-56RH was obtained. A 1% solution of CS-56RH gave no reduction of Benedict reagent.

Summary

1. In an electrophoretic fractionation of the cottonseed allergenic fraction CS-1A, a protein migrated toward the cathode and a polysaccharidic-protein migrated to the anode.

2. The evidence presented indicates that CS-1A is a mixture containing a specifically active protein and active compounds of this protein chemically combined with varying amounts of polysaccharidic carbohydrate.

3. The cutaneous activity of the cathodic and anodic fractions was not decreased by refluxing with 0.1 *N* acid for four hours.

4. The reagin neutralizing capacity of the fractions was decreased but not destroyed by the acid treatment.

5. A simple apparatus is described for large-scale, high voltage electrophoretic fractionation of water soluble ampholytes which is suitable for preparative work.

WASHINGTON, D. C. RECEIVED MARCH 17, 1941

[CONTRIBUTION FROM THE DEPARTMENT OF RESEARCH IN PURE CHEMISTRY, MELLON INSTITUTE OF INDUSTRIAL RESEARCH]

Cinchona Alkaloids in Pneumonia. IX. Quaternary Salts

BY MARY A. CLAPP, ALICE G. RENFREW AND LEONARD H. CRETCHER

Alkyl halides may be added readily to the quinuclidine nitrogen of cinchona alkaloids with the formation of quaternary salts. Reports in the literature offer rather conflicting evidence concerning the antipneumococcic action of such cinchona derivatives. The most encouraging results were the findings of Felton and Dougherty[1] that the small antipneumococcic power of dihydroquinine was enhanced in certain aromatic quaternary salt derivatives prepared by Jacobs and Heidelberger,[2,3] in particular, in dihydroquinine *p*-chloroacetylaminophenol and dihydroquinine 4-chloroacetylaminopyrocatechol. Morgenroth and Schnitzer[4] confirmed the observations of the intensified antipneumococcic action of quaternary salts of dihydroquinine with chloroacetanilide and *p*-chloroacetylaminophenol. However, similar quaternary derivatives of optochin

(1) Felton and Dougherty, *J. Expll. Med.*, **35**, 761 (1922).

(2) Jacobs and Heidelberger, THIS JOURNAL, **41**, 2090 (1919).

(3) Jacobs, "The Harvey Lectures," 1923–24.

(4) Morgenroth and Schnitzer, *Z. Hyg. Infectionskrank.*, **103**, 441 (1924).

TABLE I

BACTERIOSTATIC POWER AND TOXICITY OF CINCHONA QUATERNARY SALTS[a]

	In vitro Bacteriostasis *vs.* Pnc. II in broth at concn. of	Intraperitoneal toxicity — Deaths at dosages in mg. per 20 g. mouse					
		2	3	4	7	8	9
Hydroxyethylapocupreine dihydrochloride	1:300,000				1/30	5/30	12/30
Hydrochloride of:							
Acetanilide hydroxyethylapocupreinium chloride	1:10,000	5/30	24/30	5/5			
p-Hydroxyacetanilide hydroxyethylapocupreinium chloride	1:50,000	2/30	14/30	14/15			
p-(β-Hydroxyethoxy)-acetanilide hydroxyethylapocupreinium chloride	>1:10,000	1/30	21/30	10/10			
Dihydroquinine *p*-chloroacetylaminophenol[b]	1:50,000		19/30	27/30			
p-Hydroxyacetanilide cinchonidinium chloride	1:10,000	4/30	28/30				

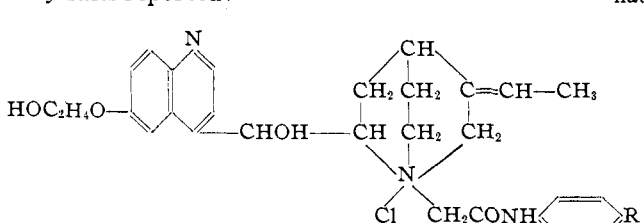
[a] The biological testing was carried out by Drs. Bracken, Patrick, Maclachlan and Johnston of the Mercy Hospital, Pittsburgh, Penna. [b] The dihydroquinine derivative was prepared in the manner described by Jacobs and Heidelberger.[2] Felton and Dougherty[1] in testing against Pnc. I had no growth at a concn. of 1:40,000 under conditions where optochin was bacteriostatic at 1:120,000; and mouse toxicity by intraperitoneal testing as given was 2.5 mg. for the largest tolerant dose. Morgenroth and Schnitzer[4] found this quaternary salt of dihydroquinine more effective against Pnc. VI but less effective than dihydroquinine against several higher-numbered types.

were found to have little antipneumococcic action despite the recognized bacteriostatic power of optochin itself.

These findings with dihydroquinine seemed to justify the study of quaternary derivatives of another cinchona alkaloid. Three quaternary salts of hydroxyethylapocupreine were prepared, but these were not bacteriostatic for the pneumococcus at dilutions above 1:50,000, whereas hydroxyethylapocupreine is effective at a dilution of 1:3 to 4×10^5. The results of tests *in vitro* and studies of mouse toxicity are given in Table I.

Because the *p*-chloroacetylaminophenol quaternary salts of dihydroquinine, optochin and hydroxyethylapocupreine were about equivalent in action *in vitro*, the corresponding cinchonidine salt was synthesized to provide a cinchona component of negligible action as a test of the contribution of *p*-chloroacetylaminophenol. But this quaternary salt was active only up to dilutions of 1:10,000.

Formula I is representative of the new quaternary salts reported:



Formula I

R = H, *Acetanilide hydroxyethylapocupreinium chloride*

R = HO, *p-Hydroxyacetanilide hydroxyethylapocupreinium chloride*

R = HOC_2H_4O, *p-(β-Hydroxyethoxy)-acetanilide hydroxyethylapocupreinium chloride*

Experimental

N-Chloroacetyl β-hydroxyphenetidine.—β-Hydroxyphenetidine was prepared by the hydrolysis of benzyloxyethyl *p*-acetaminophenyl ether[5] with 12% hydrochloric acid. Chloroacetylation of this amine was carried out in the manner described by Jacobs and Heidelberger.[6] The crude N-chloroacetyl β-hydroxyphenetidine, melting at 149 to 150°, was obtained in a 45% yield. Decolorization with charcoal and recrystallization from 50% ethyl alcohol gave a product melting at 150 to 151°.

Anal. Calcd. for $C_{10}H_{12}O_3NCl$: N, 6.10. Found: N, 5.83.

Chloroacetanilide and *p*-Chloroacetylaminophenol.—These compounds were prepared in the manner described by Jacobs and Heidelberger.[6]

Quaternary Salts.—The method of preparation was to boil equimolecular amounts of the components in dry acetone for six to seven hours as described by Jacobs and Heidelberger[2] (ten volumes of acetone were necessary for the solution of hydroxyethylapocupreine). If no reaction product separated, the acetone was removed under diminished pressure and the residue was dissolved in two volumes of absolute ethyl alcohol and filtered. The quaternary salt was precipitated from the alcoholic solution with four volumes of anhydrous ether, thus removing unreacted hydroxyethylapocupreine (which remains in the supernatant liquor).

The hydrochlorides of the quaternary salts were prepared by titrating alcoholic solutions to an endpoint acid to methyl orange.

***p*-(β-Hydroxyethoxy)-acetanilide hydroxyethylapocupreinium chloride** separated as a gum during the reaction and was obtained as an amorphous solid, after evaporation of a filtered alcoholic solution; yield 45%.

***p*-(β-Hydroxyethoxy)-acetanilide hydroxyethylapocupreinium chloride hydrochloride** crystallized from four volumes of absolute ethyl alcohol; $[\alpha]^{26}D$ −59.4° (l = 1, c = 1.009 in distilled water).

(5) Butler and Renfrew, THIS JOURNAL, **60**, 1582 (1938).

(6) Jacobs and Heidelberger, *ibid.*, **39**, 1441 (1917).

Anal. Calcd. for $C_{31}H_{38}O_6N_3Cl \cdot HCl$: N, 6.77; Cl, 11.4. Found: N, 6.6; Cl, 11.3.

***p*-Hydroxyacetanilide hydroxyethylapocupreinium chloride** separated as a gum during the reaction and was obtained as an amorphous solid in a 74% yield after the precipitate from the alcoholic solution treated with four volumes of anhydrous ether was dried.

***p*-Hydroxyacetanilide hydroxyethylapocupreinium chloride hydrochloride** was obtained as an amorphous powder by pouring an alcoholic solution of the dry hydrochloride into anhydrous ether, and crystallized from either absolute ethyl or methyl alcohol; $[\alpha]D$ $-87.6°$ ($l = 1$, $c = 0.525$ in distilled water).

Anal. Calcd. for $C_{29}H_{34}O_5N_3Cl \cdot HCl$: N, 7.29; Cl, 12.3. Found: N, 7.21; Cl, 12.1.

Acetanilide hydroxyethylapocupreinium chloride did not separate in the course of the reaction. It was obtained as an amorphous solid in a 70% yield after the alcohol–ether treatment of the acetone residue.

Acetanilide hydroxyethylapocupreinium chloride hydrochloride separated as an amorphous powder from two volumes of absolute ethyl alcohol in the course of continued heating; $[\alpha]D$ $-89.2°$ ($l = 1$, $c = 1.051$ in distilled water).

Anal. Calcd. for $C_{29}H_{34}O_4N_3Cl \cdot HCl$: N, 7.50; Cl, 12.66. Found: N, 7.50; Cl, 12.3.

***p*-Hydroxyacetanilide cinchonidinium chloride hydrochloride.**—U. S. P. cinchonidine $[\alpha]^{22}D$ $-49.9°$ ($l = 1$, $c = 1$ in pyridine) was suspended in 50 volumes of dry acetone, containing the calculated equivalent of *p*-chloroacetylaminophenol, and refluxed at 61° for fifty-two hours. A quaternary salt gradually crystallized on the walls of the flask; after twenty-four hours of refluxing the yield was 39% of theoretical, and after fifty-two hours of refluxing the yield was 57%. The quaternary salt was recrystallized from 35 volumes of absolute ethyl alcohol; $[\alpha]^{21}D$ $-30.3° \pm 0.5$ ($l = 1$, $c = 1.012$ in pyridine).

The hydrochloride of the quaternary salt was obtained as an amorphous solid, having been prepared by titrating an alcoholic solution to an end-point acid to methyl orange; $[\alpha]^{21}D$ $-47.0°$ ($l = 1$, $c = 1.085$ in distilled water).

Anal. Calcd. for $C_{27}H_{30}O_3N_3Cl \cdot HCl$: N, 8.14. Found: N, 7.93, 7.96.

Conclusion

Quaternary salts of hydroxyethylapocupreine have been prepared and tested for biological action in comparison with dihydroquinine-*p*-chloroacetylaminophenol hydrochloride.[1] The antipneumococcic action of dihydroquinine was enhanced but that of hydroxyethylapocupreine was greatly decreased, in the quaternary derivatives tested.

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[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

The Molecular Constitution of Enzymatically Synthesized Starch

BY W. Z. HASSID AND R. M. MCCREADY

Cori and Cori[1] demonstrated that animal tissues contain an enzyme, phosphorylase, which acts upon glycogen to produce glucose-1-phosphate (Cori ester). From a consideration of the structural configurations for this hexosephosphate, Cori, Colowick and Cori[2] suggested that it was α-glucopyranose-1-phosphoric acid. Later, Wolfrom and Pletcher[3] proved that the structure of the ester was the same as originally proposed by Cori and co-workers. Kiessling[4] and others[5] showed that when phosphorylases, isolated from yeast, muscle or liver, act on the Cori ester, it is transformed reversibly into a polysaccharide. Hanes[6] subsequently demonstrated that many plants contain phosphorylase which catalyzes the reversible reaction: starch + free phosphate $\rightleftarrows$ glucose-1-phosphate. According to these investigators, similar reactions occur in the breakdown and formation of glycogen or starch *in vivo*. Hanes[6b] showed that enzymatically synthesized starch is indistinguishable in certain of its characteristics from natural starch. Astbury, Bell and Hanes[7] also showed that the X-ray pattern of synthetic starch is essentially the same as that of natural starch. However, certain differences exist between the natural and synthetic starch. The latter is less soluble in water, and rapidly retrogrades in solution; it gives a more intense color with iodine and is quantitatively hydrolyzed with β-amylase to maltose. With natural starches the enzymic hydrolysis ceases when approximately 60% has been converted into maltose. This behavior toward β-amylase is similar to the amyloamylose fraction of natural

(1) C. F. Cori and G. T. Cori, *Proc. Soc. Exptl. Biol. Med.*, **34**, 702 (1936).

(2) C. F. Cori, S. P. Colowick and G. T. Cori, *J. Biol. Chem.*, **121**, 465 (1937).

(3) M. L. Wolfrom and D. E. Pletcher, THIS JOURNAL, **63**, 1050 (1941).

(4) W. Kiessling, *Biochem. Z.*, **302**, 50 (1939).

(5) C. F. Cori, *Endocrinology*, **26**, 285 (1940); *J. Biol. Chem.*, **135**, 733 (1940).

(6) C. S. Hanes, (a) *Proc. Roy. Soc.* (London), **B128**, 421 (1940); (b) **B129**, 174 (1940).

(7) W. T. Astbury, F. O. Bell and C. S. Hanes, *Nature*, **146**, 558 (1940).