[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

# Isolation and Structure of Urochloralic Acid from Urine of Calves Fed Trichloroethylene[1]

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Urochloralic acid has been isolated from urine of calves fed trichloroethylene. After hydrolysis with β-glucuronidase the aglycone was identified as 2,2,2-trichloroethanol, and the glycone as D-glucuronic acid. The structure of urochloralic acid was established by synthesis of methyl (2′,2′,2′-trichloroethyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)-uronate which had the same properties as the corresponding derivative prepared from biosynthetic urochloralic acid.

Urochloralic acid isolated in 1875[3] from the urine of men treated with chloral hydrate was one of the first[4] glycosiduronic acids recognized but its structure has never been firmly established. v. Mering[5] identified trichloroethanol as the aglycone and subsequently Butler[6] isolated trichloroethyl *p*-nitrobenzoate from acid-hydrolyzed urine of dogs which had inhaled trichloroethylene. The glycone was considered[5] to be a glycuronic acid because aqueous solutions of it were dextrorotatory and readily reduced alkaline copper solutions.[5] Moreover the analysis of its barium salt corresponded to that of D-glucuronic acid.[5] The non-reducing levorotatory[5-8] urochloralic acid upon acid hydrolysis yielded dextrorotatory solutions which gave positive orcinol and naphthoresorcinol[6] tests. Mayer and Neuberg[9] isolated the glycone of acid-hydrolyzed urochloralic acid from human urine as a *p*-bromophenylhydrazine "salt" which had the same melting point and optical rotation as a specimen prepared from D-glucuronic acid. Reference compounds from other uronic acids were not available for comparison.

Urochloralic acid, like practically all other biosynthetic glucosiduronic acids[4] with strongly negative rotation, has presumably a β-glycosidic linkage. The few α-anomers of glycosiduronic acids containing optically inactive aglycones which have been synthesized are strongly dextrorotatory.[10]

In connection with experiments on the biological effects of trichloroethylene in the bovine we had occasion to study the metabolism of trichloroethylene in this species. The urine of calves fed trichloroethylene[11] was found to contain relatively large amounts of a substance which gave a positive Fujiwara reaction[12a,b] after oxidation with a chromic oxide–nitric acid mixture. This substance was suspected of being urochloralic acid and was therefore isolated by a method previously used[7] for this purpose. Isolation of pure urochloralic acid or its salts from bovine urine appears to entail greater difficulties and greater losses than is the case with human or dog urine.[6]

After partial purification urochloralic acid was converted to methyl triacetylurochloralate, which had the same properties as a compound prepared by Smith and Williams[13] from urine of rabbits treated with trichloroethanol.

The biosynthetic urochloralic acid obtained from calves was readily hydrolyzed by bacterial or liver β-glucuronidase of which the latter has great specificity for β-glycopyranosiduronic acids.[14a,b] From such hydrolysates there were prepared: (1) 2,2,2-trichloroethyl *p*-nitrobenzoate after extraction of the aglycone with ether; (2) α-D-glucurono-6,3-lactone after purification with ion exchange resins and (3) D-glucurono-*p*-nitroanilide.

Evidence obtained from degradation that urochloralic acid is a 2′,2′,2′-trichloroethyl-β-D-glucosiduronic acid was confirmed by preparation of methyl tri-*O*-acetylurochloralate from the natural urochloralic acid. The latter had the same properties as a synthetic product obtained by condensation of 2,2,2-trichloroethanol with methyl (1-bromo-2,3,4-tri-*O*-acetyl-D-glucopyran)-uronate which afforded methyl (2′,2′,2′-trichloroethyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)-uronate.

## Experimental

**Isolation of Sodium Urochloralate.**—Following essentially the procedure of Külz,[7] filtered urine (8 l.) from a calf fed trichloroethylene (12 g. per day) was concentrated to a thin sirup in an enameled tray. The sirup was extracted exhaustively with a mixture of ethyl ether–90% ethanol–50% sulfuric acid (600:300:30 v./v.). The concentrated alcoholic extract was neutralized first with solid and finally with saturated aqueous barium hydroxide to *p*H 6.5, filtered and the filtrate treated with 10% aqueous neutral lead acetate until precipitation was complete. The precipitate was filtered and discarded; the filtrate was treated with saturated basic lead acetate until the *p*H was 8. The precipitate was centrifuged, washed and decomposed with hydrogen sulfide. The solution, freed from hydrogen sulfide, was neutralized with saturated aqueous barium hydroxide, filtered and the filtrate treated with aqueous sodium sulfate (10%) until precipitation was complete. The precipitate was filtered, washed with water and the combined filtrate and washings were concentrated *in vacuo* to dryness. The residue was extracted with absolute ethanol (15 ml.) and then with 90% ethanol (50 ml.). Sodium urochloralate was precipitated by addition of anhydrous ether, collected by centrifugation and dried *in vacuo* at 78°; yield 1.57 g.

(1) Paper No. 3415 Scientific Journal Series, Minnesota Agricultural Experiment Station, Paper No. 12 of "Studies on Trichloroethylene-extracted Feeds." Paper No. 11, see reference 11. Presented at the 128th Annual Meeting of the A.C.S., Minneapolis, Minn., September, 1955.

(2) To whom inquiries should be addressed.

(3) J. v. Mering and O. Musculus, *Ber.*, **8**, 662 (1875).

(4) H. G. Bray, *Adv. Carb. Chem.*, **8**, 251 (1953).

(5) J. v. Mering, *Z. physiol. Chem.*, **6**, 480 (1882).

(6) T. C. Butler, *J. Pharmacol. Expt. Therap.*, **97**, 84 (1949). He isolated also sodium urochloralate, $[\alpha]^{21}D$ −59°, from urine of dogs.

(7) E. Külz, *Pflügers Arch. Ges. Physiol.*, **28**, 506 (1882).

(8) H. Thierfelder, *Z. physiol. Chem.*, **10**, 163 (1886).

(9) P. Mayer and C. Neuberg, *ibid.*, **29**, 256 (1900).

(10) G. N. Bollenback, J. W. Long, D. G. Benjamin and J. A. Lindquist, THIS JOURNAL, **77**, 3310 (1955); M. Bergmann and W. W. Wolff, *Ber.*, **56**, 1060 (1923).

(11) T. A. Seto and M. O. Schultze, *Proc. Soc. Exp. Biol. Med.*, **90**, 314 (1955).

(12) (a) K. Fujiwara, *Sitzber. Abh. Naturw. Ges. Rostock*, N. F., **6**, 33 (1914); (b) T. A. Seto and M. O. Schultze, *Anal. Chem.*, submitted for publication. When heated with aqueous alkali and pyridine trichloroethylene, trichloroacetaldehyde and trichloroacetic acid but not trichloroethanol or urochloralic acid yield a red color. After oxidation with a chromic oxide–nitric acid mixture trichloroethanol and urochloralic acid give a positive Fujiwara reaction.

(13) J. N. Smith and R. T. Williams, *Biochem. J.*, **44**, 242 (1954).

(14) (a) H. Masamune, *J. Biochem. Japan*, **19**, 353 (1934); (b) G. A. Levvy and C. A. Marsh, *Biochem. J.*, **52**, 690 (1952).

of a gray amorphous solid which, analyzed by the Fujiwara reaction,[12b] had a purity of 62.5% and represented about 10% of the total trichloroethanol present in the urine.[11]

**Urochloralic Acid. A.**—Purification through lead salts: Crude sodium urochloralate (230 mg.) was purified by repeating treatment with neutral lead acetate and basic lead acetate as indicated above. The lead glucuronoside was decomposed with hydrogen sulfide, the solution decolorized with animal charcoal, and concentrated to a sirup which, after 2 days at 4°, yielded a crystalline solid (34 mg.) having m.p. 132–138°[15] (reported[7] 142°), neutralization equivalent 317 (calcd. 325.5) and contained 94% of the theoretical amount of trichloroethanol as determined by the oxidative Fujiwara procedure.[12b]

**B. Purification by Ion Exchange Resins.**—Sodium urochloralate of 37.5% purity[12b] (96 mg.) in water (25 ml.) was divided into two portions and each passed through a separate 8 × 125 mm. column of Dowex-1[16] anion exchange resin (formate form); the columns were washed successively with 15 ml. of water, 280 ml. of 0.1 *M* formic acid, 10 ml. of water and then with 0.1 and 0.2 *M* aqueous sodium bicarbonate. Progress of the retention and elution of urochloralic acid was followed by the Dische[17] carbazole reaction. The first aqueous and formic acid effluents contained appreciable amounts of chromogen which became negligible after 200 ml. of 0.1 *M* formic acid had been used. Elution of most of the chromogen occurred with the 2–5th 40-ml. portion of sodium bicarbonate. Analysis of these eluates by the oxidative Fujiwara procedure indicated that they contained the urochloralic acid. The combined eluates were concentrated to 200 ml., acidified to *p*H 4.0 with 10% formic acid, passed twice through a Dowex-50[16] column (hydrogen form), concentrated *in vacuo* to dryness and the residue dried over $P_2O_5$. The partially crystalline mass (35.3 mg.), $[\alpha]^{27}D$ −50.0 (*c* 0.7, water), did not give a sharp melting point; neutralization equivalent 352, trichloroethanol content 82.6% of theoretical.

**Methyl Triacetylurochloralate.**—Since the procedures described above had not yielded pure urochloralic acid, the crude specimens were esterified with diazomethane and then acetylated by the procedure of Smith and Williams[13] which afforded crystalline methyl triacetylurochloralate. The properties, m.p. 157–158°, $[\alpha]^{28}D$ −40.7 (*c* 1.1, chloroform), corresponded to those reported by Smith and Williams[13]; m.p. 158°, $[\alpha]^{20}D$ −37° (*c* 1.0, chloroform).

*Anal.* Calcd. for $C_{15}H_{18}O_{10}Cl_3$: C, 38.67; H, 4.11; Cl, 22.84. Found[18]: C, 38.9, 39.2; H, 4.38, 4.41; Cl, 22.8, 22.9.

**Synthesis of Methyl (2′,2′,2′-Trichloroethyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosid)-uronate.**—Methylation of α-D-glucurono-6,3-lactone (5 g.) was followed by acetylation[19,20] to yield methyl (1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranosid)-uronate (2.2 g.), m.p. 175–176°. The latter (1 g.) was brominated[21] in glacial acetic acid (4 ml.), saturated with hydrogen bromide at 0°, to yield methyl (1-bromo-2,3,4-tri-*O*-acetyl-D-glucopyranosid) uronate (0.73 g.), which after recrystallization from ether melted at 104–105°. A solution of the bromo ester (700 mg.) and 2,2,2-trichloroethanol (3 g.) in anhydrous ethyl ether (4 ml.) was shaken intermittently in the presence of silver carbonate (600 mg.) at room temperature for 48 hours. After filtration the residue was washed with chloroform, and the combined filtrate and washings were concentrated *in vacuo* at 50°. Water (3 ml.) was added to aid in the removal of excess trichloroethanol. Recrystallization of the residue first from ethyl ether and then from ethanol–water yielded methyl triacetylurochloralate (102 mg.), $[\alpha]^{25}D$ −35.6° (*c* 1.0, chloroform), m.p. 157–158°. This product showed no depression of the melting point when mixed with the methyl triacetylurochloralate prepared from calves' urine.

*Anal.* Calcd. for $C_{15}H_{18}O_{10}Cl_3$: C, 38.67; H, 4.11; Cl, 22.84. Found[18]: C, 38.7; H, 4.1; Cl, 22.6.

**Enzymatic Hydrolysis of Urochloralic Acid. A.**—Sodium urochloralate (5 mg.)[22] dissolved in water (10 ml.) was treated with either liver β-glucuronidase[23] (2500 Fishman units in 0.5 ml.) at *p*H 5.5 or bacterial β-glucuronidase[24] (2500 Fishman units in 50 mg.) at *p*H 6.5 for 12 hours at 37°. The solution was deproteinized[25] with zinc sulfate (5%, 1 ml.) and saturated barium hydroxide (1 ml.). The precipitate was centrifuged from the slightly alkaline solution (phenolphthalein) and the latter passed through a Dowex-50[16] (hydrogen form) column. The column was washed with water and the combined effluent and washings were concentrated *in vacuo* to about 1 ml. and used for chromatographic identification of the glycone.

B.—For isolation and identification of the products the enzymatic hydrolysis was carried out on a larger scale and its progress followed by the dinitrosalicylic acid reaction of Sumner.[26] Thus a partially purified specimen[22] containing 460 mg. of sodium urochloralate (oxidative Fujiwara reaction[12b]) was dissolved in water (25 ml.) and incubated at 37°, in the presence of toluene (2 drops), with liver β-glucuronidase (2 ml., 10,000 Fishman units); after 70 and 94 hours more enzyme (0.5 ml.) was added. During hydrolysis the compounds reducing dinitrosalicylic acid increased from zero at the beginning to a maximum, equivalent to 200 mg. of glucuronolactone at 94 hours; incubation for a further 24 hours did not increase the yield. After a total incubation period of 118 hours the solution was deproteinized by addition of aqueous zinc sulfate (1.5 ml., 5%) and aqueous barium hydroxide (1.5 ml., saturated). The precipitate was centrifuged, washed with water and the combined solutions were used for isolation of the cleavage products.

**Identification and Isolation of the Glycone. A. Chromatographic Identification.**—The eluate obtained from enzymatic hydrolyzates of urochloralic acid were chromatographed by descending technique with two different systems: (1) butanol-1–acetic acid–water (4:1:5 v./v.)[27] was used with Whatman No. 1 paper; after spraying with the *p*-anisidine–trichloroacetic acid reagent of Hough, *et al.*,[28] two red spots with $R_f$ values identical with those of authentic specimens of D-glucuronic acid ($R_f$ 0.15) and D-glucurone-6,-3-lactone ($R_f$ 0.30) were obtained, corresponding closely to those reported by Partridge[27]; (2) propanol-1–water (89:28 v./v.) was used with alginic acid-treated Whatman No. 1 paper[29]; after spraying with the *p*-anisidine reagent[26] the following colors and $R_f$ values of unknown and authentic specimens were observed: D-glucose, brown, 0.34; D-galacturonic acid, red, 0.33; D-mannuronic acid, red, 0.41; D-glucurono-6,3-lactone, red, 0.35 and 0.49; acid-hydrolyzed D-glucurono-6,3-lactone, red, 0.35 and 0.49; enzyme or acid hydrolyzed urochloralic acid, red, 0.35 and 0.48; acid hydrolyzed methyl triacetylurochloralate 0.35 and 0.48. The two spots are the result of partial lactonization of the glucuronic acid, the latter having the lower $R_f$ value.

B. Isolation of D-Glucurono-6,3-lactone.—The hydrolyzate (30 ml.) from the enzymatic cleavage of urochloralic acid, containing reducing sugar equivalent to about 200 mg. of glucuronolactone was divided into 4 fractions and each passed through a 8 × 100 mm. column of Dowex-1 (formate form) anion exchange resin. The effluents were tested with the dinitrosalicylic acid reagent.[26] The columns were successively washed with water (15 ml.), 0.01 *M* formic acid (30 ml.), 0.1 *M* formic acid (40 ml.) and 0.2 *M* formic acid (30 ml.) the last removing most of the reducing sugar from the resin. The combined fractions containing the reducing material were concentrated to dryness under reduced pressure at 50°. A chromatographic test indicated a predominance of free glucuronic acid in the residue. To promote lactonization, absolute ethanol (5 ml.) was added to the residue, distilled off and the residue heated *in vacuo* at 75° for 3 hours. The residue, which now consisted mostly of D-glucurono lactone, was dissolved in warm methanol, and treated with ethyl ether (3 ml.). The precipitate was centrifuged and discarded; the supernatant was evaporated to dryness in a stream of dry air and dried *in vacuo* at 78° over $P_2O_5$; yield 205 mg. The product (100 mg.) was dissolved

(15) Melting points determined with Fisher–Johns block.
(16) A product of the Dow Chemical Co., Midland, Mich.
(17) Z. Dische, *J. Biol. Chem.*, **183**, 489 (1950).
(18) Analysis by Clarke Microanalytical Laboratory, Urbana, Ill.
(19) O. Touster and V. H. Reynolds, *J. Biol. Chem.*, **197**, 863 (1952).
(20) W. F. Goebel and F. H. Babers, *ibid.*, **100**, 743 (1933).
(21) W. F. Goebel and F. H. Babers, *ibid.*, **111**, 347 (1935).
(22) 62.5% purity as determined by Fujiwara reaction.[12b]
(23) Warner Chilcotte Laboratories, New York, N. Y.
(24) Sigma Chemical Co., St. Louis, Mo.
(25) M. Somogyi, *J. Biol. Chem.*, **160**, 69 (1945).
(26) J. B. Sumner, *ibid.*, **65**, 393 (1925).
(27) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).
(28) L. Hough, J. K. N. Jones and W. H. Wadman, *J. Chem. Soc.*, 1702 (1950).
(29) D. R. Spriestersbach, Ph.D. Thesis, University of Minnesota, 1954.

in glacial acetic acid (15 ml.), treated with charcoal and filtered. Upon cooling, crystals of D-glucurono-6,3-lactone separated. These were filtered, washed successively with cold glacial acetic acid, absolute ethanol and anhydrous ether; yield 82 mg., $[\alpha]^{23}D$ +19 (*c* 3.3 in water), m.p. 171–172° and mixed m.p. with an authentic specimen 172–174°.

*Anal.* Calcd. for $C_6H_8O_6$: C, 40.92; H, 4.6. Found[30]: C, 40.9; H, 4.9.

**C. D-Glucurono-6,3-lactone-*p*-nitroanilide.**—Recrystallized-D-glucurono-6,3-lactone (20 mg.) obtained from enzymatic hydrolysis of urochloralic acid and recrystallized *p*-nitroaniline (22 mg.) were heated at 78° for 20 minutes with methanol (0.6 ml.) containing 0.07 ml. of concd. hydrochloric acid per 100 ml. as suggested by Spriestersbach.[29] After cooling, the *p*-nitroanilide separated as fine needles; it was collected by centrifugation, washed with cold methanol and recrystallized from methanol. The product (9 mg.), dried over $P_2O_5$, had m.p. and mixed m.p. with an authentic specimen of 129°.

*Anal.* Calcd. for $C_{12}H_{13}O_7N_2$: N, 9.46. Found[30]: N, 9.1.

Descending chromatography of *p*-nitroanilides on Whatman No. 1 paper was carried out with a solvent system of hexanol-1–concd. ammonium hydroxide–water (10:0.5:50, v./v.), the aqueous layer being the moving, the organic layer the stationary phase. $R_f$ values of the yellow spots of different compounds were: *p*-nitroaniline, 0.48; D-glucose-*p*-nitroanilide,[31] 0.75; D-glucurono-6,3-lactone-*p*-nitroanilide (authentic specimen), 0.86; D-glucurono-6,3-lactone-*p*-nitroanilide from urochloralic acid, 0.85.

**Isolation and Identification of Aglycone.**—Application of differential analyses for trichloroacetic acid and trichloroethanol by the modified Fujiwara reaction[12b] showed that the biosynthetic urochloralic acid contained a component which reacted like trichloroethanol and not like trichloroacetic acid or trichloroacetaldehyde.

A deproteinized enzymatic hydrolyzate (30 ml.) prepared as described above containing an estimated amount of 50 mg. of trichloroethanol was saturated with sodium chloride and extracted three times with a total of 27 ml. of ethyl ether and the extract dried over anhydrous magnesium sulfate. Analyses of the ether extract by the oxidative Fujiwara procedure indicated that it contained only 23 mg. of trichloroethanol. After removal of the ether with a stream of dry air under slightly reduced pressure the residue was heated with *p*-nitrobenzoyl chloride (35 mg.) and dry pyridine (1 ml.) on a steam-bath for 40 minutes. The mixture was cooled to room temperature and diluted dropwise with water (1 ml.). After 30 minutes the solution was added dropwise to a mixture (10 ml.) of sodium bicarbonate (10%) and ice chips. The crystals which separated were centrifuged, washed with water and dried in a desiccator over $P_2O_5$. Recrystallization from petroleum ether (b.p. 70–80°) yielded a product (10 mg.), m.p. 69–70°, and mixed m.p. 69–70° with an authentic specimen of 2,2,2-trichloroethyl *p*-nitrobenzoate.

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(30) Huffman Microanalytical Laboratories, Wheatridge, Colo.

(31) F. Weygand, W. Perkow and P. Kuhner, *Ber.*, **84**, 594 (1951).

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[CONTRIBUTION FROM DEFENCE RESEARCH CHEMICAL LABORATORIES]

# Amino Acids. II. Synthesis of Cyclic Guanidino Acids[1]

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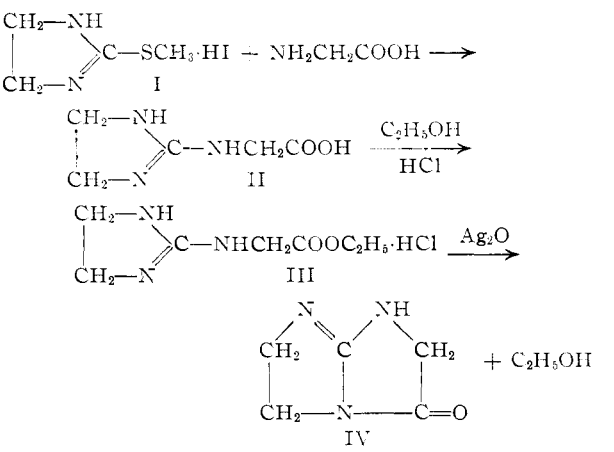
A new series of N-substituted amino acids has been synthesized by the reaction of amino acids with S-methyl cyclic isothioureas. Some derivatives of these acids are described together with the bicyclic compounds 3-keto-$\Delta^8$-hexahydro-1,4,8-pyrimidazole and 3-keto-2,3,5,6-tetrahydro-1-imidaz[1.2-a]imidazole.

A new series of cyclic guanidino acids analogous to those described by Elderfield and Green[3] has been synthesized. These acids (Table I) were prepared by allowing a sodium or potassium salt of an amino acid to combine with a S-methyl cyclic isothiourea at room temperature. Further characterization was effected by converting them to the picrates of their ethyl esters.

Although the acids could be purified by crystallization, the yields (25–50%) were very poor, owing to the similarity in solubility of the guanidino acids and the iodide salts. However, the acids could be recovered readily in good yield by passing the total aqueous reaction mixture through a mixed-bed of IRA-400 and IRC-50 resins which removed the potassium or sodium iodide salts completely, leaving the cyclic guanidino acids in solution.

2-Methylmercapto-2-imidazolinium iodide (I) and glycine in sodium hydroxide solution gave 2-(carboxymethylamino)-$\Delta^2$-1,3-diazacyclopentene (II). This acid was then esterified to 2-(carbethoxymethylamino)-$\Delta^2$-1,3-diazacyclopentene hydrochloride (III) and identified as its picrate. When the ester hydrochloride was shaken with silver oxide in water, a mixture of the bicyclic compound 3-keto-2,3,5,6-tetrahydro-1-imidaz[1,2-a]imidazole (IV) and the free acid 2-(carboxymethylamino)-$\Delta^2$-1,3-diazacyclopentene (II) was obtained. The bicyclic compound IV was only isolated in the pure




(1) Issued as D. R. C. L. Report No. 170.

(2) Monsanto Canada Limited, Ville LaSalle, Quebec.

(3) R. C. Elderfield and M. Green, *J. Org. Chem.*, **17**, 442 (1952).