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# ISOLATION OF CITROSAMINE FROM CITRUS LEAVES*

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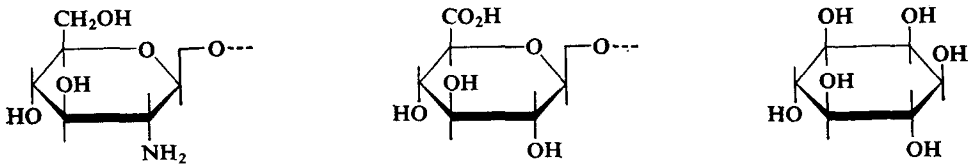
**Abstract**—A nitrogen containing trisaccharide was found in citrus leaves, identified as glucosamido-glucuronido-inositol and given the trivial name citrosamine.

## INTRODUCTION

DURING an investigation of nitrogenous constituents of citrus leaves, a ninhydrin reactive substance was isolated and given the trivial name citrosamine. This paper describes the procedures used in the isolation and identification of this compound. Citrosamine (I) was found to be composed of glucosamine, glucuronic acid, and inositol. It was isolated from "Dancy" tangerine leaves and apparently occurs in the free form. Carter *et al.*[1] have reported a similar compound obtained by hydrolysis of phytoglycolipid from phosphatides found in seeds of corn and flax. A comparison of i.r. spectrum of the compound from corn with that from citrus indicated that the two were similar in structure but not identical. The main difference was in the region between 8 and 10 $\mu$ (Fig. 1). Repeated crystallization did not change the spectra.



(I)

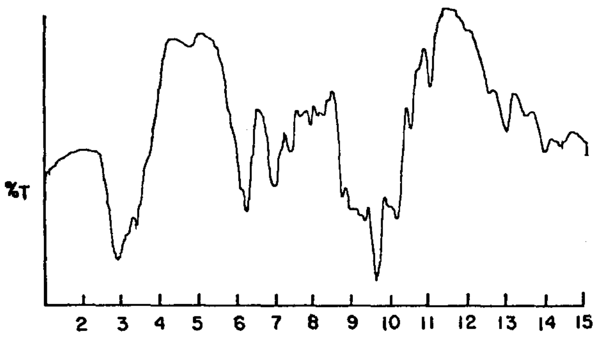


FIG. 1. INFRARED SPECTRUM OF CITROSAMINE.

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* Florida Agricultural Experiment Stations Journal Series No. 2934.

[1] H. E. CARTER, S. BROOKS, R. H. GIGG, D. R. STROBACH and T. SUAMI, *J. Biol. Chem.* **239**, 743 (1964).

Hydrolysis of citrosamine with HCl gave glucosamine and inositol, but no hexuronic acid could be isolated. Glucosamine was identified chromatographically and by conversion to arabinose with ninhydrin. Inositol was identified by comparing the i.r. spectrum of the isolated material with that of an authentic myo-inositol sample.

Although no hexuronic acid was found after acid hydrolysis, the presence of an uronic acid was indicated by ester formation, a positive Dische test,[2] the failure of citrosamine to move on paper electrophoresis at pH 7·4, and strong absorption peaks at 6·22 and 7·0 $\mu$. Kent and Whitehouse[3] have pointed out that during acid hydrolysis of carbohydrates containing uronic acids, the acid may decompose before the glycosidic linkages are cleaved. Therefore, nitrous acid degradation was employed,[1] which gave glucuronido-inositol. Mild hydrolysis of this compound gave glucuronic acid and inositol. The glucuronic acid was identified by chromatography. The glucuronic acid also was reduced to glucose which was identified chromatographically and by a specific reaction with glucose oxidase.

Neither citrosamine nor glucuronido-inositol reduced Fehling's solution indicating that bonding involved the aldehyde carbons of both glucosamine and glucuronic acid. The point of attachment of the other bonding to glucuronic acid and inositol is not known.

## EXPERIMENTAL

### *Extraction and Isolation*

Leaves from "Dancy" tangerine trees were dried in a triple pass, hot flue gas dryer for approximately 10 min at 68°. The dried leaves were ground in a Wiley mill to pass a 60-mesh screen. Twenty-seven kg of dried leaves were stirred for 2 days with 178 l. of 50 per cent ethanol. Extractions were also carried out by grinding freshly frozen leaves in a Waring blender with 50 per cent methanol. In both methods, the alcohol was removed under vacuum at 50° and the remaining water extract taken to a small volume. Pigments and other extraneous material were extracted into ether and discarded.

The water extract was then passed through a Dowex 50W × 8 resin ($H^+$) followed by a wash with 50 per cent ethanol and finally with water. The amino-containing compounds were then eluted from the resin with 2N $NH_4OH$ and eluate evaporated under vacuum at 50° until a thick dark syrup formed. The syrup was placed on a 14 × 16 cm column of Whatman CF 11 cellulose powder. A mixture of 1-butanol:acetic acid:water (108:27:40) was passed through the column and collected in 500 ml fractions. An aliquot of each fraction was chromatographed overnight on Whatman No. 1 filter paper using the above solvent. Citrosamine had an $R_f$ of 0·04. The greatest concentration of citrosamine was found in fraction 36. Fractions containing the compound were reduced in volume until needle-shaped crystals of citrosamine formed along with a flocculent precipitate. Both the crystals and precipitate were collected, dissolved in water, and treated with sufficient Darco G-60 charcoal to remove color. The precipitate was removed by adding ethanol to the clarified solution. Additional ethanol brought about crystal formation of citrosamine. Repeated crystallization separated the crystals from the precipitate as indicated by paper chromatography using water-saturated phenol as a solvent and ninhydrin for an indicator.

*Identification.* Citrosamine did not have a well-defined melting point but turned brown at approximately 235°. (Found: C, 38·13; H, 6·82; N, 2·58. Calc. for $C_{18}H_{31}O_{16}N \cdot 2H_2O$: C, 39·05; H, 6·33; N, 2·53 per cent). Van Slyke N, 2·91 per cent. Molecular weight, cryoscopic, 553. $[\alpha]_D + 119$ (7·14 per cent in $H_2O$). Citrosamine (10 mg) was refluxed in 2 ml 6N HCl. Samples were taken at intervals and chromatographed on Whatman No. 1 filter paper with the solvent 1-butanol:pyridine:water:benzene (50:30:30:4·5) (BPWB). Three spots were detected using either ammoniacal silver nitrate or a periodate-permanganate-benzidine reagent.[4] The parent compound disappeared with time until none was found after 1 hr. Substances with the chromatographic characteristics of glucosamine and inositol formed as the parent compound disappeared.

*Identification of glucosamine.* In addition to the paper chromatographic method above, glucosamine was identified by co-chromatography with the authentic compound on a Technicon amino acid analyzer (retention time relative to norleucine of 0·69). Glucosamine was also identified by oxidative degradation with

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[2] Z. Dische, *J. Biol. Chem.* **167**, 189 (1947).

[3] P. W. Kent and M. W. Whitehouse, *Biochemistry of the Aminosugars*, p. 61. Academic Press, New York (1955).

[4] R. J. Block, E. L. Durrum and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd edition. Academic Press, New York (1958).

ninhydrin to form arabinose.[5] The citrosamine hydrolyzate (1 ml) and an excess of ninhydrin were put into a boiling water bath for 15 min and compared with an authentic sample of glucosamine treated in a similar manner. Both samples gave chromatographic spots (solvent BPWB) with identical $R_f$ values to arabinose and different from lyxose which is the oxidative product of galactosamine.

*Identification of inositol.* Citrosamine (100 mg) was refluxed in 2 ml 6 N HCl for 1 hr. The HCl was removed by concentration to dryness three times with $H_2O$ in a flash evaporator at 50°. The dry residue was put into a vacuum dessicator overnight with KOH. The hydrolyzate was dissolved in water, passed through a Dowex 50W ($NH_4^+$) resin column, and then through a Dowex 2 ($HCO_3^-$) resin column. The fraction that passed through both columns was concentrated to a small volume (about 5 ml) and then streaked on Whatman 3 MM paper. Strips of the chromatogram corresponding to the area for inositol were cut into small pieces, extracted with water in a Waring blender, and filtered. The filtrate was reduced to a small volume, and the inositol precipitated with abs. ethanol and ether. The precipitate was centrifuged and dried under vacuum (yield 12 mg). The i.r. spectrum of the degraded material was identical to that of authentic myo-inositol.

*Identification of glucuronic acid.* Ethyl ester formation. Citrosamine (10 mg) was refluxed for 1 hr in 5 ml 10 per cent anhyd. HCl in ethanol. Most of the HCl was removed by concentration to dryness several times with ethanol. The ester was precipitated from ethanol with ether. It showed a strong peak at 5·7 $\mu$ in the i.r. On paper electrophoresis (phosphate buffer[4] pH 7·4, 450 V for 2 hr), the ester moved 3·0 cm toward the cathode while citrosamine showed no movement.

For the isolation of glucuronic acid, citrosamine (100 mg) was dissolved in 10 ml water containing 83 mg $NoNa_2$. The solution was adjusted to pH 3·0 with acetic acid and stirred overnight at room temperature. The solution was then passed through a CG 120($H^+$) resin column and evaporated to less than 1 ml. Ten ml of abs. ethanol and 10 ml of ether were added and the precipitate filtered. The precipitate was dissolved in a few ml of water and the precipitation procedure repeated. The resulting precipitate was dried overnight under vacuum (yield 59·5 mg). The precipitate was chromatographed on paper using the BPWB mixture. One spot was obtained at $R_f$ 0·02. Citrosamine showed no movement. The precipitate (10 mg) from the nitrous acid degradation was refluxed with 5 ml of 3N HCl for 3 hr. The hydrolyzed material was taken to dryness twice with water and then dried overnight in a vacuum dessicator over KOH. The residue was dissolved in 2 ml of water and chromatographed on paper. There were three spots—two of these corresponding to glucuronic acid, one being the lactone. The third spot was identical with inositol. This indicated that the degradation product from nitrous acid was glucuronido-inositol. Further studies were carried out to positively identify glucuronic acid since chromatographic evidence alone is usually not sufficient.

*Reduction of glucuronic acid to glucose.* The methyl ester was formed by refluxing glucuronido-inositol (50 mg of the crude precipitate) in 12·5 ml of 2·2 per cent anhyd. HCl in methanol and allowing this solution to stand overnight at room temperature. The solvent was removed under vacuum, and the methanolic HCl treatment repeated. The solvent was concentrated to a small volume and the ester precipitated with ether. The precipitate was filtered and dried (yield 33·6 mg). The crude product was very hydroscopic.

The carboxyl group of glucuronic acid was reduced to an alcoholic group by dissolving the crude methyl ester of glucuronido-inositol (33·6 mg) in 10 ml of water and adding dropwise 10 ml of 0·05 M $NaHCO_3$ containing 50 mg of $NaBH_4$. After remaining overnight, the mixture was neutralized with acetic acid and passed through a CG 120 ($H^+$) resin column and then through a Dowex 2 ($HCO_3^-$) resin column. The solution was concentrated to a small volume and precipitated with benzene and ethanol. The precipitate was very hydroscopic. A few mg were collected on a funnel and dissolved in 0·5 ml N HCl and passed into a small tube. The tube was sealed and heated to 100° for 2 hr. The hydrolyzate was chromatographed on paper using the BPWB solvent system. Two spots were found, and these corresponded to glucose and inositol. Glucose was further identified by a specific glucose oxidase test.[6] The hydrolyzate (0·1 ml) was compared with a glucose standard, fructose, and a water blank. The glucose oxidase test gave a color reaction for both glucose and the hydrolyzate with maximum absorption at 525 nm. There was no color formation with fructose or the blank. The reduction of the hexuronic acid to glucose is evidence that glucuronic acid is a constituent of citrosamine.

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[5] P. W. Kent and W. M. Whitehouse, *Biochemistry of the Aminosugars*, p. 204, Academic Press, New York (1955).

[6] Fermco Laboratories, Inc., Chicago. Tech. Bull., *Enzymatic Determination of Glucose* (1964).