N_{100} values of 52 and 32, the former being somewhat greater in quantity. These values are lower by 7 than those obtained with capsorubin, indicating the probable presence of four hydroxyl groups, with one in a less hindered position than the 6 or 6′ positions in reduced capsorubin. After treatment of the reduced product with hydrochloric acid in methanol, the N_{100} of the main product in system II was 73 (the product obtained from reduced capsorubin had a value of 74). Apparently, it is a diol. It appears that IV-5 is a 6′-keto-3,3′,4′-triol.

The apparently undescribed carotenoids IIIA-1, IIIB-1, IIIB-2, IIIB-4, IIID-1, IIID-2, IV-1, and IV-5, as well as the substances tentatively identified as capsanthin-5,6-epoxide and capsochrome, were all present as minor constituents (0.4 to 2.3% of the total carotenoids) which will render more difficult the complete elucidation of their structures.

Seven components were found to contain ketone groups, capsanthin, capsorubin, cryptocapsin, capsanthin-5, 6-epoxide-like, capsochrome-like, P-441 (tetrahydrocapsorubin ?), and polyol IV-5.

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SUGAR CANE PHOSPHOLIPIDES

The Isolation, Separation, and Identification of the Principal Phospholipides of Sugar Cane Juice

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This investigation of the phospholipides of sugar cane juice is part of a broad study on nonsugars in cane juice. Because of the emulsifying nature of phospholipides, it is believed that these compounds have a deleterious effect on the processing and clarification of cane juice. By utilizing a combination of solvent fractionation and silicic acid column chromatography, the principal phospholipides were isolated from lyophilized fresh sugar cane juice and separated. They corresponded chromatographically to phosphatidyl ethanolamine and lecithin on silicated glass paper.

Determination of the composition of the total lipide fraction of sugar cane juice has been the object of numerous investigations, primarily because of interest in sugar cane wax which has commercial value. Balch (*2*) has compiled a fairly complete bibliography of the subject up to 1953. The phospholipide fraction of the total lipides has no commercial value as a by-product, but is thought to be of importance to the sugar industry because of the deleterious effect that phospholipides would have upon the formation and settling of the precipitate during clarification of the juice. Honig (*8*) points out that, since lecithin and other phosphatides are well known emulsifying agents, even small quantities of these substances in sugar cane juice would affect clarification adversely. Despite the apparent importance of these compounds, a review of the literature reveals a paucity of information. Shorey (*13*) undertook a study of a wax-like solid that he obtained from sugar cane juice in 1897; he found that this substance contained both phosphorus and nitrogen, and had the physical properties of lecithins. In another early study of sugar cane lipides, Wijnberg (*14*) reported the presence of lecithin in sugar cane wax. More recently, Hatt, Strasser, and Troyahn (*7*), also investigating the wax fraction of sugar cane, reported that the presence of glycerol and phosphorus could be accounted for by a phosphatide fraction. Their evidence, however, was indirect as they did not isolate phospholipides as such, nor did they identify the nitrogen-containing radicals usually associated with phospholipides. Honig's (*8*) study suggests that the phospholipide in sugar cane juice is a lecithin, but he points out that definite proof of its presence is still lacking.

In view of the scarcity of information on this important constituent of sugar cane juice, this investigation was undertaken as part of a broad study on the composition of nonsugars in cane juice.

Methods

Preparation of Material for Analysis. Samples of fresh, raw sugar cane juice were obtained from grinding individual varieties of cane under commercial conditions in the experimental Audubon Sugar Factory at Louisiana State University during pilot plant clarification studies as described by Guilbeau, Black, and Martin (*4*). The fresh juice was frozen and dried on a large capacity lyophilizer as reported by Roberts (*12*). Mixed lots of the dried juice, in quantities varying from 500 to 1000 grams, were extracted in a 1-gallon capacity blender using an ethanol–ethyl ether (2 to 1, v./v.) solvent system; the dried juice and solvent were blended at medium speed for approximately 5 minutes and were then allowed to stand for 3 hours. This mixture was filtered under vacuum through a large, coarse-

porosity, fritted-glass filter funnel. The filter cake was washed with the solvent mixture and then re-extracted twice in the blender; all filtrates were combined and evaporated on a rotary vacuum apparatus until the solvent was removed and the lipides had taken on the consistency of a thick, dark green oil. This oily residue was dissolved in ethyl ether and extracted with several portions of water to remove water-soluble contaminants, such as sugars and organic acids. The ether fraction was decanted, and then further freed of water by treatment with anhydrous calcium chloride, after which the ether was removed under vacuum in a rotary evaporator.

The mixed lipides were dissolved in as small a quantity of chloroform as practical and this solution was added to 10 volumes of acetone and placed in a freezer at −10° C. for several hours, or overnight. Under these conditions, a lipide fraction precipitated which contained the phospholipides; the bulk of the lipide material and pigments remained dissolved in the acetone. While still cold, the insoluble lipides were removed from the acetone by centrifuging for approximately 10 minutes, at 3000 r.p.m.; the precipitated lipides were washed with cold acetone once, redissolved in a small quantity of chloroform, and then reprecipitated as before. This purified phospholipide preparation served as the starting material for all subsequent analyses and chromatographic separations.

Hydrolysis and Evaluation of the Hydrolyzate. Samples of the mixed phospholipide preparation were subjected to methanolic–hydrochloric acid hydrolysis as suggested by Artom (*1*). The water-soluble portion of the hydrolyzate was analyzed by standard paper chromatography; two solvent systems which were used were 1-butanol–acetic acid–water (250:60:250, v./v./v.), and phenol saturated with water. Ninhydrin, 1% in acetone or methanol, used as a dip or spray, indicated the presence of amino acids and other ninhydrin-reactive components. Preparations of pure amino acids and ethanolamine were used as reference compounds. The hydrolyzate was analyzed for choline with Reinecke acid as suggested by Glick (*3*).

Column Chromatography. Large-scale separation of phospholipides was accomplished by a column chromatographic technique modeled after that of Hanahan (*6*). This method utilized a silicic acid–Hyflo Super-Cel column, mixed in a ratio of two to one, respectively. A 60-gram column of the mixture was found satisfactory. The amount of phospholipide added to the column—usually a total of 2 to 5 mg. of phospholipide phosphorus—was always considerably less than the 0.8 to 1.0 mg. per gram of silicic acid, as suggested by Hanahan, but this in no way seemed to affect the performance of the columns. The columns were eluted with a mixture of chloroform and methanol applied in the following dilutions (v./v.): (I) 7 to 1, (II) 4 to 1, (III) 3 to 2, and (IV) 1 to 4. After several exploratory runs using these solvent systems, it was found that one or more of the dilutions could be eliminated, reducing the number of tubes collected without interfering with the separation. The fractions were collected on an automatic fraction collector using a siphon which delivered 8 ml. per tube. To locate the phospholipides as they were eluted from the column, an appropriate aliquot was removed from each tube, the solvent evaporated on a water bath, and the lipides digested with concentrated sulfuric acid and 50% hydrogen peroxide; the residue was diluted with water and analyzed for phosphorus by the method of King (*9*).

Glass-Paper Chromatography. The phospholipides separated by the column technique were identified by the silicated glass-fiber paper method of Muldrey (*11*). Glass-fiber paper, freed from organic contaminants by heating in a muffle furnace at 1100° F. for 0.5 hour, was dipped in a 1% potassium silicate solution and dried at 110° C. The chromatograms were developed in a solvent system consisting of 100 parts of pyridine, 100 parts of benzene, and varying amounts of water between 5 to 15 parts. All solvents were of the highest quality available, and were used without further purification. The spots on the developed chromatograms were detected by applying a fine mist of concentrated sulfuric acid to the dried glass paper strips with a nebulizer, and then heating over an open coil heater until charring occurred and the spots developed. To aid in identifying the unknown phospholipides, various reference compounds were used. Inositol phosphatide, phosphatidyl ethanolamine, phosphatidyl serine, and several lecithins were purchased from a biochemical supply house. Lecithin and phosphatidyl ethanolamine were also readily prepared from an egg, as suggested by Lea and Rhodes (*10*). This egg preparation of phosphatidyl ethanolamine proved to be the most reliable source, as the commercially available compounds were very impure.

Results and Discussions

The water-soluble portion of the hydrolyzate of the mixed phospholipides was subjected to paper chromatography in an attempt to identify the nitrogen moiety of the phospholipide molecule. It was possible to obtain ninhydrin reactive spots which had an R_f value corresponding to that of ethanolamine; certain preparations produced a second ninhydrin reactive spot which matched that of serine. None of the preparations, however, produced very reliable results. The spots were always quite diffuse and faint, and could not be reproduced consistently. All hydrolyzate preparations, however, produced a positive choline reaction when tested with Reinecke acid, even under the specific conditions of producing a precipitate in an alkaline medium. Since the chromatographic tests on the hydrolyzate were not decisive, consideration was given to chromatography of the intact phospholipides, utilizing silicic acid.

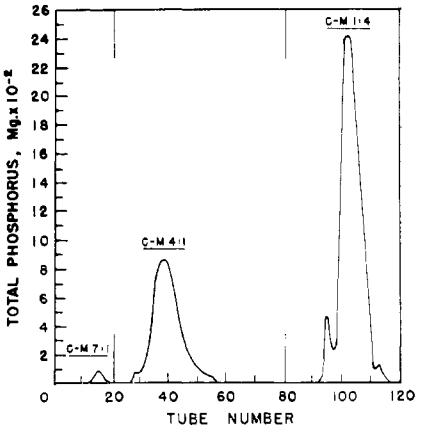

Figure 1. Chromatogram of sugar cane juice phospholipides on a silicic acid–Hyflo Super-Cel column

Eluting solvents were mixtures of chloroform and methanol in ratios (v./v.) indicated above the fractions. The two major peaks are identified on the glass-paper chromatogram shown in Figure 2

The partially purified phospholipide preparation was dissolved in approximately 10 to 15 ml. of solvent mixture (I) to obtain a solution containing 2 to 5 mg. of phospholipide phosphorus which was added to the column. Progressively, the four solvent mixtures mentioned were used to elute the column. This produced 200 tubes on the average; however, many of the tubes were blank, and there was a large spread between phosphorus peaks. Further runs, eliminating solvent mixture (III), 3:2 v./v. chloroform-methanol, achieved adequate separation and only 150 tubes or less were required. The results of a typical run are shown graphically in Figure 1, where tube number is plotted against milligrams of phosphorus. Most of the phospholipide phosphorus can be accounted for in two major peaks; the small peak which appears between tubes 10 to 20 was evident in all runs, but accounts for less than 1% of the phosphorus. This phosphorus component closely followed the solvent front, and was eluted with a relatively large quantity of yellow-pigmented lipide material. The small quantity of phosphorus-containing lipide and relatively large quantity of the other lipide material in this

Figure 2. Positive print made from a contact negative of a glass-paper chromatogram showing the chromatographic identification of the principal phospholipides in sugar cane juice

component hindered all efforts to identify it. Another small phosphorus-containing component appeared in a few runs between the two major peaks, but because of its inconsistency and trace characteristic, no attempt was made at identification.

Several runs, in which all four solvent mixtures were used, produced what appeared to be a third major peak closely associated with the second component coming from the column. This proved to be an artifact, however, produced by the introduction of solvent mixture (IV) just as the second major phosphorus component was being eluted by solvent (III). Apparently, the introduction of this more polar solvent system at this point caused a more rapid elution of the component, producing a third peak. Eliminating solvent (III) and starting solvent (IV) at approximately tube 80 eliminated this trouble and produced a curve with a much steeper slope. On most runs, it was possible to account for 85 to 90% of the phosphorus added to the column; the balance apparently remained on the column. The faster moving component accounted for one third, and the slower component for two thirds of the phospholipide phosphorus eluted from the column; these ratios were consistent for all runs.

The chromatographic identification of the two major components was accomplished on silicated glass-fiber paper with corresponding reference compounds as described by Muldrey (*11*). After the tubes containing the phospholipides had been located by analyzing aliquots from each tube for phosphorus, the tube contents of each zone were composited, and the solvent evaporated from each component. These were taken up in chloroform and spotted on the silicated paper along with various reference compounds. The solvent system found most reliable for separating the components consisted of 100 parts of pyridine, 100 parts of benzene, and 5 parts of water. Figure 2 is a positive print made from a contact negative of a chromatogram showing the spots produced from the unknown components along with their identifying reference compounds. *A* and *C* represent the fast and slow moving major components, respectively, which were collected from the column. *B* represents reference compounds phosphatidyl ethanolamine near the origin, and lecithin the spot farthest from the origin. One component in *A* corresponds chromatographically to phosphatidyl ethanolamine; the single spot in *C* corresponds to lecithin. The other large spot or mixture of spots in *A* near the solvent front represents several nonphospholipide contaminants which always seem to be eluted from the column along with phosphatidyl ethanolamine. Several attempts to isolate the phosphatidyl ethanolamine from these contaminants by the use of additional silicic acid column proved unsuccessful. Since this spot or mixture of spots turned pink to purple on gentle heating after sulfuric acid spraying, it is suspected that steroids are present (*5*). It is also possible that any free fatty acids which may occur in the preparation would also be carried near the solvent front as indicated by Muldrey, Miller, and Hamilton (*11*).

Since the hydrolyzate experiments indicated the possible presence of serine, it was hoped that the silicated-glass paper chromatography studies would reveal the presence of phosphatidyl serine. The spot on the origin in fraction *A* (Figure 2) was believed to contain phosphatidyl serine, along with possible degradation products. To move this component from the origin, the polarity of the solvent system was increased by addition of more water. In this instance, a spot was obtained between phosphatidyl ethanolamine and the origin, which according to Muldrey and coworkers (*11*), would be the proper order for phosphatidyl serine on silicate paper. Subsequent runs with a reference phosphatidyl serine, however, did not produce coinciding R_f values. Since this spot was not identified as phosphatidyl serine, other phosphatides were investigated; inositol phosphatide, lysolecithin, and sphingomyelin were chromatographed along with fraction *A*, but none produced corresponding R_f values. This relatively slow moving spot is either some unidentified phospholipide or a degradation product of one of the other components in this group.

No conclusion can presently be drawn regarding phosphatidyl serine, since this compound, in trace quantities, may possibly occur in association with the phosphatidyl ethanolamine fraction (*6*). The concentration required to produce a spot for phosphatidyl ethanolamine may be too low to reveal phosphatidyl serine with the technique employed. It is concluded from these studies that the two principal phospholipides of sugar cane correspond chromatographically to phosphatidyl ethanolamine and lecithin. The chromatographic methods employed do not distinguish lecithins or phosphatidyl ethanolamines which differ in the particular fatty acids with which the two nonphosphate groups of the glycerol are esterified. Larger quantities of these constituents would have to be isolated and purified to establish their structures completely, and for elemental analyses necessary for positive chemical identification; however, the physical properties and physicochemical behavior of phosphatides that might be expected to affect the processing of juice and crystallization of sugar are common to all of the compounds of this class.

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