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SUGAR OCCURRING IN THE EXTRAFLORAL EXUDATES OF THE ORCHIDACEAE*

STEVEN I. BASKIN† and CARMAN A. BLISS‡

Department of Pharmacognosy, School of Pharmacy, University of Southern California, California 90007

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Abstract—The sugar content of the extrafloral nectars of thirty orchid species representing twenty genera has been studied by paper and gas chromatography. Fructose, glucose and sucrose were found in all nectars while the presence of raffinose and other oligosaccharides were indicated. The relative α- and β-glucose contents of the nectars were also determined.

INTRODUCTION

THE ORCHIDACEAE, possibly the largest and most highly evolved[1,2] family in the angiosperms, contain from 20,000 to 35,000 species distributed among 500–600 genera. The family is notable because of the large number of genera excreting both intrafloral and extrafloral nectars (exudate)[3] but the chemical composition of the floral nectars has been little studied. Because of the possible importance of these substances in the physiology of the plant,[4–8] a study of the carbohydrate composition of the extrafloral nectars would seem to be of value.[3,9–11]

Sucrose, glucose and fructose are the most common carbohydrates found in plant nectars. Mucilage, protein, organic acids[12,13] and water-soluble vitamins[14] are also found. Semi-quantitative analysis of sugars in various plant nectars from a large number of plant families

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† This paper was submitted by Steven I. Baskin in the 1966 Lunsford Richardson Undergraduate Research Competition and was awarded first place in Region I. National Science Foundation Undergraduate Research Participation Fellow 1965–1966 and National Institutes of Health Trainee (GM 1417) 1967–Present. Present address: The Ohio State University, College of Medicine, Department of Pharmacology, Columbus, Ohio 43210.

‡ Present address: University of Saskatchewan, College of Pharmacy, Saskatoon, Saskatchewan, Canada.

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was achieved by Wykes in 1952[6] and by Percival in 1960[15] with the use of paper chromatography. Fructose, glucose, sucrose, maltose, melibiose and raffinose were found in varying amounts. Percival[15] analyzed the nectar of eight orchid species, but indicated that only one was an extrafloral exudate. This exudate contained glucose, fructose, sucrose and raffinose. In May 1965, Chen Chuan Tu[16] reported glucose, fructose, sucrose and a fourth more complex saccharide in the extrafloral nectar of *Cattleya* "Estelle" × *C. intermedia* var. *alba*, Cowan. With the exception of these two preliminary studies,[15, 16] all previous work has been carried out on intrafloral exudates.

The nectar-excreting glands or nectaries may be grouped into extrafloral nectaries (outside but close to the flower) or intrafloral nectaries.[3, 17, 18] The physiology of nectar secretion has been studied.[6, 9–11] Darwin, in 1862,[19] was one of the first to describe nectar secretion in orchids. Sunding[3] and others[6] have pointed out differences between extrafloral and intrafloral nectaries in the orchid family. Differences in function, if they exist,[3, 6, 10] may possibly be indicated by differences in the sugar composition.

Previously, paper chromatography has been the main means of identifying the sugars of nectars.[15, 16] Further refinement of the identification techniques is possible utilizing gas–liquid chromatography because of its greater sensitivity and versatility in detecting mono-, di- and trisaccharides. A combined procedure utilizing paper and gas–liquid chromatography provides a superior method for identification of carbohydrates in minute samples of nectar, and has been employed in the present work.

RESULTS AND DISCUSSION

Paper Chromatographic Studies

Initially, the sugar content of several of the extrafloral nectar samples were examined by paper chromatography, using the procedure of Hough *et al.*[20] The R_g values of the sugars contained in each sample were compared with those of reference sugars and were identified as fructose, sucrose and glucose. Small amounts of raffinose and a fifth unknown sugar were also found in some of the samples examined (Table 1). The relative concentration of each sugar was subjectively determined by the color intensity and the size of the sugar spot, after development, when examined under u.v. light (360–400 nm). Although this procedure is only an approximate one, differences in relative sugar concentrations between samples could be detected (see Table 1).

Gas Chromatography Studies

The use of Yamakawa and Ueta's[21] modification of Sweeley's basic method[22] avoided the problems of the tailing the pyridine peak and of the syringe clogging with ammonium chloride. Initial gas–liquid chromatography studies were carried out on known sugar

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TABLE 1. SUGARS IDENTIFIED IN EXTRAFLORAL NECTARS BY PAPER CHROMATOGRAPHY

Orchid species	Fructose	Glucose	Sucrose	Raffinose	Unknown
Aërides falcatum, Lindl. & Paxt.	3*	3	3		
Ansellia africana, Lindl.	3	3	1		
× *Brasso-Cattleya* "Nanipuakea"	2	2	4		
Catteyopsis lindeni, Lindl.	2	3	2		
Cattleya bowringiana, Veitch	2	2	3		
Cymbidium aloifolium, Wall	3	3	3		
Cyrtopodium punctatum, Lindl.	2	3	3		
Dendrobium chrysotoxum, Lindl.	3	3	3	1	
Epidendrum anza, E. W. McLellan	3	3	3	1	
E. atropurpureum, Hemsl.	2	3	3	1	1
Laelia tenebrosa, Rolfe	2	2	4	2	1
× *Laelio-Cattleya* "Adolph Hecker"	2	2	3	2	
× *L.-C.* "Walter Armacost"	2	2	4		
Phalaenopsis lueddemanniana, Reichb. f.	1	3	3	1	
Sobralia, Ruiz & Pav. sp.	2	3	2		
Vanda rothschildiana, Chassaing	1	2	2		

* Relative amounts of sugars are shown by the following: 1 = trace amount, 2 = small amount, 3 = medium amount and 4 = large amount.

TABLE 2. GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF STANDARD SUGARS

Sugar	Retention values (cm) at 205°			
Xylose	6·6 (m)*	7·3		
Ribose	6·1			
Ribitol	7·5			
Dulcitol	13·4			
Sorbitol	13·3			
Glucose†	11·5	14·2		
Galactose	9·5 (s)	10·4 (m)	11·8	
Fructose	8·3			
Rhamnose	8·0	10·3 (s)		

Sugar	Retention values (cm) at 250°			
Fructose	0·9 (t)	1·1 (m)	2·2 (s)	
Sucrose	10·1			
Glucose	1·2 (s)	1·4 (m)	2·2 (t)	
Maltose	10·5 (t)	12·8 (m)	14·2 (t)	15·2
Cellobiose	10·6 (m)	12·0 (s)	13·9	15·0
α-Lactose	14·6 (m)	16·3 (t)	16·7 (t)	
β-Lactose	10·3 (s)	14·1 (m)	17·9 (s)	
Trehalose	14·0			
Melibiose	17·6			
Turanose	12·4			
Gentiobiose	19·8			
Raffinose	74·7			

* The following abbreviations are used in describing the peaks: (t) = trace, (s) = small, (m) = major.

† Multiple peaks with specific sugars correspond to the anomeric forms of these sugars.[22]

derivatives and their retention values were determined (Table 2). Separation and determination of retention values were carried out under isothermal conditions at 205 and 250°. A column temperature of 205° gave good resolution of monosaccharides while a column temperature of 250° was necessary for the elution of the oligosaccharides. Confirmation of the major monosaccharide peaks in nectars was made by separation at a column temperature of 205° and comparing the retention values with those of standard sugars. These monosaccharide peaks could then be easily correlated with the corresponding peaks which occurred when the samples were chromatographed at 250°.

The retention values of both monosaccharides and disaccharides contained in the nectars were determined (Table 3) and compared with those of standard sugars at 250°. Monosaccharides of the nectars were also compared with those of standard sugars at 205°.

TABLE 3. GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF MONO AND DISACCHARIDES FROM NECTAR

Orchid species	Retention values (cm) at 250°			
Aërides falcatum, Lindl. & Paxt.	0·9 (t)*	1·1	9·7 (m)	12·8 (t)
Ansellia africana, Lindl.	1·2 (m)	2·2	2·6 (s)	10·1
× *Brasso-Cattleya* "Nanipuakea"	1·3	1·7	10·3 (m)	17·9 (t)
Catteyopsis lindeni, Lindl.	2·0 (s)	2·6	9·9 (m)	17·7 (t)
Cattleya bowringiana, Veitch	2·0 (s)	2·5 (s)	10·0 (m)	
Cyrtopodium punctatum, Lindl.	2·1 (s)	2·6	9·6 (m)	13·2 (t)
Dendrobium chrysotoxum, Lindl.	0·9 (s)	2·2 (s)	10·4 (m)	
Epidendrum anza, E. W. McLellan	2·1 (s)	2·6 (s)	11·4 (m)	74·7 (t)
E. atropurpureum, Hemsl.	2·2	2·6	11·6 (m)	74·7 (t)
Laelia tenebrosa, Rolfe	2·1 (s)	2·6	9·4 (m)	13·3 (t)
× *Laelio-Cattleya* "Adolph Hecker"	2·1	2·6	8·8 (m)	74·7 (s)
× *L.-C.* "Walter Armacost"	1·7	2·6 (s)	9·7 (m)	14·7 (t)
Odontoglossum cariniferum, Reichb. f.	7·4	3·6	10·4 (m)	
Phalaenopsis lueddemanniana, Reichb. f.	0·7	1·5	2·6 (s)	10·0 (m)
Sobralia, Ruiz & Pav. sp.	1·1	2·2 (s)	10·4 (m)	18·3 (t)
Vanda rothschildiana, Chassaing	0·7 (s)	1·0	9·5 (m)	13·2 (t)
Zygopetalum intermedium, Lodd.	1·1 (m)	2·2 (s)	10·3	

* The following abbreviations are used in describing the peaks: (t)=trace, (s)=small, (m)=major.

By increasing the sensitivity of the chromatograph and enlarging the volumes injected, several oligosaccharides occurring in trace concentrations were detected. These were not positively identified, but from comparative retention values of known sugars they were tentatively identified as maltose, cellobiose, gentiobiose, raffinose and lactose. The occurrence of extraneous peaks in both standard sugar and in nectar samples made a careful examination and interpretation of the chromatographs necessary. The silanization procedure involving the silanization in pyridine solution and the subsequent evaporation of the pyridine gave rise to the formation of multiple peaks in certain sugars.[23] By careful standardization of technique and by comparison of the sugars in the unknown samples with those of standard sugars most extraneous peaks could be accounted for and identification of the true sugar peaks could be made.

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For a quantitative comparison of the sugars within each nectar, the silanized samples were chromatographed utilizing linear temperature programming. Satisfactory separation was obtained at a temperature rise of 6° per min. The relative quantity of each sugar was determined by calculating the peak area produced by the sugar and comparing this area with that of sucrose. Values were then determined as a per cent value of sucrose, as determined by the formula:

$$\frac{\text{peak area sugar}}{\text{peak area sucrose}} \times 100 = \% \text{ sucrose} \pm 5\%$$

TABLE 4. RELATIVE QUANTITATIVE AMOUNTS OF SUGARS FROM THE ORCHID NECTAR

Orchid species	Fructose	α-Glucose	β-Glucose	Total glucose	Sucrose	Raffinose
Ansellia africana, Lindl.	215	230	295	525	100	
× *Brasso-Cattleya* "Nanipuakea"	25	20	30	50	100	
× *Brasso-Laelio-Cattleya* "Estelle Sewall"	50	195	45	240	100	
Cattleya aurantica, P. N. Don "Mexico"	40	35	45	80	100	
C. bowringiana, Veitch	40	80	35	115	100	
C. "Estelle" × *C. intermedia*, var *alba*, Cowan	40	30	45	75	100	
Cattleyopsis lindeni, Lindl.	155	220	200	420	100	
Chysis laevis, Lindl.	50	35	60	95	100	
Cycnoches chlorochilon, Lindl.	20	25	40	65	100	
Cyrtopodium punctatum, Lindl.	1265	90	85	175	100	
Cymbidum "Showgirl"	60	165	210	375	100	
C. aloifolium, Wall	20	10	15	25	100	
C. canaliculatum, R. Br.	170	100	115	215	100	
Diacrium bicornutum, Beth.	25	10	15	25	100	
Epidendrum "Stella"	30	15	25	40	101	
E. anza, E. W. McLellan	60	55	60	115	100	10
E. atropurpureum, Hemsl.	80	250	170	420	100	
Laelia tenebrosa, Rolfe	55	55	65	120	100	10
× *Laelio-Cattleya* "Adolph Hecker"	60	50	65	115	100	15
× *L.-C.* "Walter Armacost"	25	25	25	50	100	
× *L.-C.* "Paradiso"	35	20	25	45	100	0·5
× *Miltonidium* "Surprise"	105	40	50	90	100	
Odontoglossum "Finest"	5	15	15	30	100	
O. cariniferum, Reichb. f.	15	10	15	25	100	5
Phalaenopsis lueddemannian, Reichb. f.	125	120	135	255	100	5
Renanthera "Tom Thumb"	10	40	45	85	100	
Sobralia, Ruiz & Pav. sp.	305	255	275	530	100	
Trigonidium obtusum, Lindl.	35	30	30	60	100	
Vanda rothschildiana, Chassaing	35	40	45	85	100	
Zygopetalum intermedium, Lodd.	75	535	150	685	100	

The major sugars in the nectars of the thirty species examined were sucrose, α-glucose, β-glucose and fructose. Raffinose was found as a significant peak in five species (Table 4). Of interest is the variation in the amounts of each sugar. Relative sucrose concentrations, for example, varied widely. A consistent anomaly in the sucrose peaks was the presence of minor side peaks on each of the major peaks. A second anomalous minor peak occurred between α- and β-glucose in all temperature programmed samples. These anomalous peaks

were not seen when the nectar samples were prepared by the method of Sweeley *et al.*[22] but did occur with the method of Yamakawa and Ueta.[21] Also of interest is the occurrence of raffinose in two of three × *Laelio-Cattleya* examined (Table 4). Furthermore, the presence of raffinose in the one *Laelia* species tested and its concomitant absence from a *Cattleya* species (*C. bowringiana*) suggests that raffinose content of the nectar may be controlled by a single dominant gene.

The ability of the gas chromatographic method to determine α- and β-glucose (see Table 4) provides a further refinement over previous methods of examination of nectars, although under the experimental conditions, the differences in peak heights of the two anomers are probably not significant in most samples. Differences in the amounts of sugars were found to vary from nectar to nectar but in most cases a relatively constant ratio of fructose:glucose:sucrose was found. Of thirty species tested, twenty orchids contained mostly sucrose. In general, the sugar content of the extrafloral orchid nectars resembles that of the limited number of intrafloral orchid nectars examined by Percival.[15] This similarity in carbohydrate content opens to question the supposedly different physiological and biochemical functions of these two types of nectars.[3] It was noted that during the collection of samples that one genus, *Paphiopedilum*, showed no extrafloral nectar secretions. The absence of the nectar secretion by this genus leaves the true physiological function open to question.

EXPERIMENTAL

Paper Chromatographic Procedure

Separation of sugar standards and sugars in nectar was accomplished by the method of Hough *et al.*[20] utilizing descending chromatography on Whatman No. 1 paper and ethyl acetate–pyridine–water (8:2:1). The separated sugars were detected with *p*-anisidine HCl. Approximately 15 μg of material was spotted on the paper for analysis.

Collection and Storage of Nectar

Nectar was collected from the base of the sepals of the flowers by means of fine glass capillaries and stored under vacuum at 5°. The samples were collected at selected greenhouses within a 100 mile radius of Los Angeles between the months of May 1965 and February 1966.

Preparation of Material for Gas Chromatography

10 mg of either standard sugars or nectar, dissolved in 1 ml of pyridine was treated with 0·3 ml hexamethyldisilazane (HMDS) and 0·1 ml of trimethylchlorosilane (TMS), then shaken vigorously for 20 sec and the mixture heated for 3 min at 60–70°. Since certain of the lower molecular weight sugars could not be detected because of the pyridine solvent peak, the method of Sweeley *et al.*[22] was modified[21] as follows: 3 ml $CHCl_3$ were added to the reaction mixture above, the solution was shaken and 3 ml of distilled water were added. The mixture was again shaken and the aqueous-pyridine phase discarded. This extraction procedure was repeated three times. The $CHCl_3$ solution remaining was evaporated to dryness. The residue was then taken up in $CHCl_3$; 2-ml volumes were prepared for the standard sugar samples, while volumes of 50–200 μl were prepared for unknown exudate samples.

Analysis of the trimethylsilyl derivatives was performed on an F & M 810 Research Gas Chromatograph equipped with hydrogen flame ionization detector and dual coiled copper-alloy columns, 6 ft by 0·25 in. o.d. The columns were packed with 3% SE-30 on Chromasorb W 80–100 mesh. Flow rates were in the range of approx. 75 ml per min with inlet pressures of around 17 lb/in². The pressures of nitrogen, hydrogen and air were 40, 14 and 20 lb/in², respectively. For isothermal operation the column temperature was maintained at 250° with flash heater and detector at 260 and 270°, respectively. Linear temperature programmed analyses were accomplished by adjusting the program rate to 6° per min, holding at isothermal condition when 290° was attained. Sample volumes varied from 0·3 to 1·5 μl depending upon sugar concentration in the sample. Under isothermal conditions the chart speed was set at ½ in/min; while under linear temperature program conditions the chart speed was set at ¼ in/min.

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