Gymnemic Acid, the Antisaccharine Principle of *Gymnema sylvestre*. Studies on the Isolation and Heterogeneity of Gymnemic Acid A_1

George P. Dateo, Jr.,* and Louis Long, Jr.

The antisweet activity of *Gymnema sylvestre* leaves resides in gymnemic acid, a mixture of triterpene saponins of closely related structure. Gymnemic acid A_1 has been isolated by others and described as the major active component. A gymnemate salt fraction was isolated from an aqueous ethanol extract of dried leaves by solvent extraction. Chromatography on high-porosity, quaternary ammonium resin (acetate form) with 1 *N* acetic acid in ethanol-water (7:3) furnished an A_1-rich fraction. Preparative thin-layer chromatography (silica gel, BuOH-H_2O, NH_3 atmosphere) gave A_1 identical in chromatographic behavior with samples from other laboratories. Gymnemic acid A_1 was resolved into components A_{11} and A_{12} by thin-layer chromatography (silica gel, $CHCl_3$-*t*-BuOH-HOAc-H_2O, 8:2:2:1). Both A_{11} and A_{12} were found to be active and to be distinguishable by nmr and uv spectral properties.

As early as 1848 (Falconer, 1848), the potential importance of the antisweet effect of *Gymnema sylvestre* leaves was appreciated, in that it "might, consequently, lead to some important physiological discovery as regards the organ of taste." Gymnemic acid (Figure 1), the antisaccharine principle of the leaves of *Gymnema sylvestre*, is a mixture of triterpene saponins (Stöcklin, 1969b). The major constituents are β-D-glucuronides of differently acylated gymnemagenins. The structure 3β,16β,21β,22α,-23,28-hexahydroxy-olean-12-ene has been assigned to gymnemagenin (Rao and Sinsheimer, 1968, 1971; Stöcklin, 1969a). The aglycone of gymnemic acid A_1 is reported to be esterified with formic, acetic, isovaleric, and tiglic acids (Sinsheimer *et al.*, 1970).

The difficulties that may attend isolation of pure gymnemic acids are illustrated in the studies of the crystalline main saponin "aescin" of the horse chestnut (*Aesculus hippocastanum* L.), isolated as a crystalline mixture of possibly 30 different substances. The problem is complicated by the lability and migration of acyl groups on the polyhydroxy triterpene aglycone (Wagner *et al.*, 1970; Wulff and Tschesche, 1969). Protoaescigenin, the main triterpene of aescin, is a stereoisomer (inverted at C-4 and C-16) of gymnemagenin.

References to recent studies on gustation involving the taste-modifying properties of gymnemic acid are given by Faull and Halpern (1971). Mindful of the reported lability of similar triterpene saponins, we have endeavored to prepare gymnemic acid extracts for psychophysical studies under isolation conditions less conducive to artefact formation than those previously employed.

EXPERIMENTAL SECTION

Materials. Dried leaves of *Gymnema sylvestre* were obtained from the Himalaya Drug Distributing Agency, Bombay, India (June 1967). A reference sample of gymnemic acid A_1 (90% pure) was supplied by T. Reichstein (Stöcklin *et al.*, 1967). J. E. Sinsheimer furnished samples of gymnemic acids A and B and of the "ethyl acetate acids" (A, B, C, and D; Sinsheimer *et al.*, 1968). A commercial gymnemic acid sample was obtained from K & K Laboratories, Inc.

Ether (dried over Na-Pb alloy), acetone (dried over molecular sieves), and diethyl carbonate were distilled before use. Absolute alcohol was used. Dodecanol suppressed foaming of aqueous solutions and was removed by ether extraction. Distilled water for taste studies was air-equilibrated.

Apparatus. Rotary evaporation under vacuum at a bath temperature of <30° required condensation at Dry Ice temperature. Weights of fractions were calculated from the residue weight of a sample volume with a Cahn Electrobalance. Samples were dried under vacuum over silica gel. Melting points (uncorrected) were taken on a Nalge-Axelrod hot stage. Spectra were obtained on the Beckman IR9 and Cary 14 or 15 spectrophotometers, and on the Varian HA 100 nmr spectrometer (internal standard, tetramethylsilane). Acetone-d_6 served to prepare 5–10% solutions of the gymnemic acids. D_2O was added to detect hydroxyl proton absorption. Uv absorptivity (a) data are calculated in units of l. g^{-1} cm^{-1}. The uv spectra are for the solvent 5% ethanol (v/v).

Chromatography. Plates (20 cm) for thin-layer chromatography (tlc) were prepared from Merck silica gel G or purchased from Analtech, Inc. (Merck silica gel GF, 250 μ). Plates were air dried and not activated by heat. Fractionations were monitored by tlc with samples (10 to 30 μg) of 0.5 to 2% solutions in MeOH, $CHCl_3$-EtOH (2:1), or acetone. Spotting of equal weights facilitated comparison. Spots were made visible by a modified Lieberman-Burchard reagent (Belic, 1956). Plates were sprayed with Ac_2O-$CHCl_3$ (1:1) and H_2SO_4, and then heated on a hot plate (surface temperature, 150°). The following solvent systems were used for ascending development: I, BuOH-HOAc-H_2O (4:1:5, upper layer); II, BuOH saturated with H_2O, chamber equilibrated with 1.5 *N* NH_3; III, butyl formate-methyl ethyl ketone-HCOOH-H_2O (5:3:1:1); IV, $CHCl_3$-*t*-BuOH-HCOOH-MEOH (4:1:1:1); V, $CHCl_3$-*t*-BuOH-HOAc-H_2O (8:2:2:1); and VI, $CHCl_3$-MeOH (9:1). Stability of components during development was tested by two-dimensional chromatography with the same solvent system (Stahl, 1969).

Preparative thin-layer chromatography (ptlc) was conducted on 20 × 20 cm plates (2 mm, silica gel GF, Analtech) prewashed with $CHCl_3$-EtOH (1:1), the developing solvent, and the eluting solvent, EtOH-H_2O (8:2), acidified with 2 vol % of HOAc. Excised zones were chromatographically eluted. Dry column chromatography (dcc, see Loev and Goodman, 1970) was performed with silica gel (Bio-Sil A, Bio-Rad Laboratories) which had been dried at 120° overnight and then equilibrated with 15% water. Sample loads were prepared by plating on silica gel from acetone and then drying the silica under vacuum. Ion-exchange gels [AG1-X2 (200–400 mesh), Bio-Rad; Sephadex QAE A-25, Pharmacia] were recycled and changed to the appropriate counterion form, in the manner recommended by suppliers. Anion exchangers in acetate form were equilibrated with 3% acetic acid in EtOH-H_2O (7:3) to repress hydrolysis. Columns were operated at a linear flow rate of 10 to 20 cm hr^{-1} with the solvent EtOH-H_2O (7:3). Concentrates of fractions were freed of residual acetic acid by dilution with ethanol-water (8:2) and reconcentration.

Pioneering Research Laboratory, U. S. Army Natick Laboratories, Natick, Massachusetts 01760.

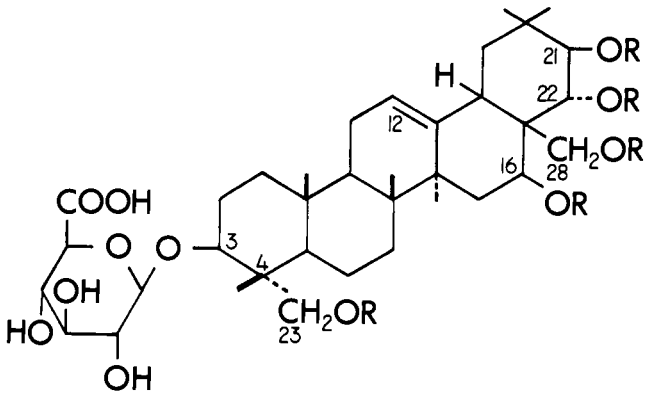

Figure 1. Gymnemic acid, R = H or acyl.

Gymnemate Salt Extract. Dry leaves of *G. sylvestre* (1558 g) in powdered form (Wiley mill) were divided into six portions. Each portion was agitated on a reciprocal shaker with 1.5 l. of EtOH-H_2O (2:1) for 16 hr at 30°. After filtration, extractions were repeated three times each with 1 l. of EtOH-H_2O (3:2) for 2 hr. The combined extracts (24 l.) were concentrated to 1.5 l. The aqueous concentrate was extracted four times with hexane (equal volumes) and four times with diethyl ether (equal volumes). The defatted aqueous phase was then extracted four times with 1.5 l. of $CHCl_3$-EtOH (2:1). The combined $CHCl_3$-EtOH phase was dehydrated by treatment with 10% Na_2SO_4 (50 ml/l.). The syrup obtained on concentration to dryness was dissolved in ethanol (400 ml). The precipitate formed at 5°, by addition of 3 to 4 vol of ether, was collected by centrifugation and dried to yield 66.3 g of a tan nonhygroscopic powder, which was soluble in water, ethanol, and acetone.

Anion-Exchange Chromatography (aec). Gymnemate salts (15.3 g), dissolved in EtOH-H_2O (7:3, 150 ml), was applied to a column (bed volume, 724 ml, 48 × 4.4 cm i.d.) of AG1-X2 acetate, equilibrated with 0.1 *N* HOAc in the solvent EtOH-H_2O (7:3). Elution was effected with EtOH-H_2O (7:3, 2.1 l.) and 1.0 *N* HOAc in EtOH-H_2O (7:3). Fractions (fr) of 20 ml were collected. Fr 165-245 contained 1.76 g of material rich in A_{11} (tlc A_{11}, A_{12}, B_1) and fr 266-295 (1.45 g) (tlc A_{12}, B_1) were reserved for isolation of A_{12}.

Gymnemic Acid A_1. From a gymnemate salt extract (3 g), a 1.41-g fraction containing A_1 and B_1 was obtained by aec on AG1-X2 acetate. Ptlc (system II) of 285 mg furnished 158 mg of gymnemic acid A_1. Aec purification (AG1-X2 acetate, 40 ml) gave 112 mg of A_1. Soxhlet extraction with ether of material plated on Celite yielded, on concentration, 84 mg of gymnemic acid A_1 melting at 202°. The uv spectrum exhibited a shoulder at 220 nm (a 7.6).

Methylation of Gymnemic Acid A_1. A_1 (9.6 mg) in MeOH (0.5 ml) was treated with ethereal diazomethane at 5°. The solution was evaporated to dryness and redissolved in acetone for tlc investigation.

Gymnemic Acid A_{11}. Fr 165-245 (1.67 g) obtained by aec of gymnemate salts (15.3 g-run) were rechromatographed on AG1-X2 acetate (396 ml) to yield 1.22 g (tlc A_{11}, A_{12}, B_1). *Via* ptlc (triple development, system V), 150 mg furnished 64 mg of homogeneous A_{11}. Aec [QAE-Sephadex acetate, 64 ml, 18.5 × 2.1 cm i.d., 10-ml fractions; elution with EtOH-H_2O (140 ml) and 1 *N* HOAc] gave fr 30-39 containing A_{11} (25.3 mg). Extraction with diethyl carbonate (50°) yielded, on evaporation, an amorphous gel. An acetone solution was clarified by filtration (Millipore Solvinert filter, 1.5 μ) for nmr. A sample for uv analysis was prepared by ether extraction from cellulose powder. Concentration gave a colorless amorphous precipitate.

Gymnemic Acid A_{12}. Dcc (silica gel, 60 g, system V) of 103 mg of fr 266-295 from aec furnished A_{12} (35.2 mg). Crystallization from ethyl carbonate-acetone yielded A_{12} (8 mg) melting at 208°; uv spectrum, shoulder at 220 nm (a 5.1).

Conversion of A_{11} to A_{12}. A forefraction from a large-scale isolation of gymnemic acid by aec (53 g of gymnemate salts on a 540-ml bed column of AG1-X2 acetate) was reserved as a potential source of A_{11} (tlc A_{11}, B_1, A_{12} absent). The material (1.2 g) was converted to ammonium salts (1.00 g) by Dowex 50-X8 (NH_4^+ form, 30-ml bed; solvent, 50% EtOH) with no change in gymnemic acid composition. Dcc of 858 mg on two columns (silica gel, 200-325 mesh, each 27 × 4.5 cm i.d., system V) yielded 158 mg of material free of B_1. Rechromatography (two runs, silica gel, 20-44 μ, each 31 × 314 cm i.d.) yielded 70 mg of A_{12} material. Further purification by aec (QAE-Sephadex acetate) and Soxhlet extraction with ether furnished 23.3 mg of A_{12}, mp 201° (undecomposed at 300°). The nmr spectrum was identical to that of previously isolated A_{12}.

Antisaccharine Activity. Solutions of gymnemic acid were prepared by adding sufficient 0.1 *N* $KHCO_3$ (10 μl/mg) to a suspension in water. Subjects (2-3) were offered a series of sucrose solutions ($\frac{1}{320}$-$\frac{1}{5}$ *M*), each differing in concentration by a factor of 2. The subject reported that solution which first tasted sweet. The subject rinsed with water between tasting the solutions. The subject then held a gymnemic acid solution in his mouth with constant tongue movement for 1 min. The solution was ejected and the mouth rinsed for 1 min. The subject's threshold solution was redetermined (within 5 min). The maximum ratio of threshold concentration after gymnemic acid to concentration before, or the elevation in threshold, was usually 4, but one subject with a high threshold had a measure of 8 (*cf.*, Warren and Pfaffmann, 1959).

RESULTS AND DISCUSSION

The isolation of the gymnemate salt fraction was partly patterned after the procedure of Khastgir *et al.* (1958), who found the antisaccharine principle of *G. sylvestre* could be extracted from water by chloroform-ethanol. The material so isolated has been utilized in taste studies (Bartoshuk 1969, 1970; Bartoshuk *et al.*, 1969; see also Meiselman and Halpern, 1970). In view of indications that the active principle was a saponin (Yackzan, 1966) and a glucuronide (Pfaffmann, 1959), we utilized tlc systems I and II and a Lieberman-Burchard reagent to monitor further separation. Anion-exchange chromatography (aec) with quaternary ammonium gels in the acetate form and acetic acid as eluent was performed in aqueous alcohol to take advantage of partition and molecular sieve separation factors (Samuelson, 1967). The alcohol increases solubility of gymnemic acid in the mobile phase. The method also avoids mineral acid precipitation.

In preliminary aec experiments, it was established that the antisweet activity was confined to fractions eluted by 1 *N* acetic acid and that the fractions contained components made visible by Lieberman-Burchard reagent (Dateo and Bartoshuk, 1967).

Heterogeneity of Gymnemic Acid A_1. After publication of the discoveries of the Reichstein group (Stöcklin *et al.*, 1967), we directed our efforts toward facile isolation of gymnemic acid A_1, reported to be the major active component. Table I summarizes the tlc nomenclature of gymnemic acid and relates our findings to those of Sinsheimer's group (Rao and Sinsheimer, 1971, and earlier references) and Kurihara (1969), and to the original work of Stöcklin *et al.* (1967). Gymnemic acid A_2, postulated to be an artefact, was not detected by us, even with application of the isolation procedure of Stöcklin *et al.* (1967).

Gymnemic acid A_1 was isolated as outlined in Figure 2.

Table I. Tlc Nomenclature of Gymnemic Acids

Stöcklin *et al.* (1967)[a]	Sinsheimer *et al.* (1968)[b]	Kurihara (1969)[e]	Present investigation
A_1	A[c]	A_1	A_1 (A_{11} + A_{12})
A_2	B[c]		
	C[d]		
	D[d]		
A_3		A_3	B_1 (B_{11} + B_{12})
A_4			

[a] Gymnemic acid A was resolved into four components, named in order of decreasing R_f (tlc, system III). [b] Antisaccharine activities have not been reported. Minor constituents V, W, X, Y, and Z have been isolated (Sinsheimer *et al.*, 1970). [c] A and B are reported identical to A_1 and A_2, respectively. [d] Characterized as glycosides of gymnestrogenin (Sinsheimer and Rao, 1970). [e] Identified by tlc (system III) with a preparation of gymnemic acid A (A_1–A_4) by the procedure of Stöcklin *et al.* (1967).

Table II. Thin-Layer Chromatography of Gymnemic Acid on Silica Gel G

	R_f × 100[a] in Solvent system[b]					
	I	II	III	IV	V[c]	VI
A_1 (A_{11} + A_{12})	77	28	40	63 58	18 (32) 13 (27)	
Diazomethane-treated A_1					47 33	41 28
B_1	68	17	12		4 (18)	

[a] R_f × 100 values are to be taken as guide values and are not reproducible. [b] See Experimental Section. [c] Values in parentheses are more representative.

It melted at 202° to a slightly yellow viscous fluid [lit. 279–283° dec (Sinsheimer *et al.*, 1970); 285° dec (Stöcklin *et al.*, 1967); 205° dec (Kurihara, 1969)]. Tlc with solvent systems I, II, and III showed chromatographic identity with a sample of gymnemic acid A_1 from Reichstein's group. The developer, IV, recommended by Sinsheimer *et al.* (1968) was tried, but under conditions different from those reported by Sinsheimer *et al.* (1970). The gymnemic acid was resolved into two components, A_{11} and A_{12} (A_{11} in lesser amount). Two-dimensional tlc ruled out transformation of A_1 into two components during chromatography. Our resolution of A_1 is attributable to the use of a smaller sample size and to the observation (de Zeeuw, 1970) that tlc in an unsaturated chamber with multicomponent solvent systems may yield improved separations. Reproducibility of R_f is sacrificed for the advantage of a vapor gradient, a phenomenon that has developed into vapor-programmed tlc.

Gymnemic acid A_1 samples from other laboratories were resolved by tlc (system IV) into A_{11} and A_{12}. Further confirmation ensued from tlc of the methyl glucuronides formed by reaction with diazomethane. Tlc data are summarized in Table II. B_1 is resolvable into B_{11} and B_{12} by multiple or continuous development (Stahl, 1969) with system V. It is tentatively identified as A_3 by comparison with a commercial sample which appeared to contain mainly gymnemic acid A_2 accompanied by A_1, A_3, and A_4 (tlc, system III).

Isolation of Gymnemic Acids A_{11} and A_{12}. Reexamination of the gymnemate salt extract by tlc established the presence of A_{11} and A_{12} prior to anion-exchange separation. Solvent system V was designed for application to preparative thin-layer chromatography (ptlc) and checked by two-dimensional tlc for stability of the acids during development. A renewed isolation of gymnemic acid by aec was monitored by tlc with systems I, II, and V (Figure 3). An A_{11} fraction (with lesser amounts of A_{12} and B_1) was enriched by repetitive aec. Ptlc (multiple development, system V) and purification by aec (Sephadex QAE) yielded 25 mg of amorphous A_{11}. From an A_{12} fraction (lesser amount of B_1) dry column chromatography on silica gel with system V furnished material homogeneous in A_{12} from which A_{12} (7.6 mg), mp 208° (undecomposed at 300°), was obtained.

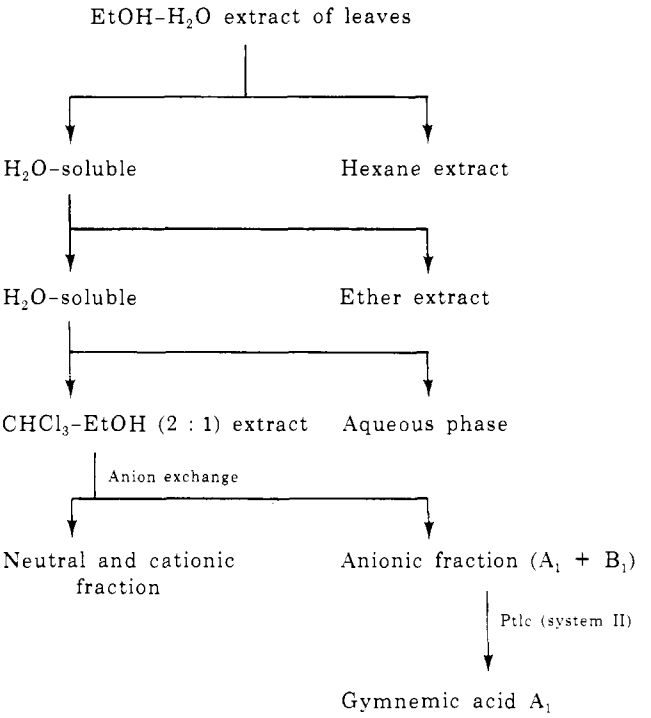

Figure 2. Isolation of gymnemic acid A_1.

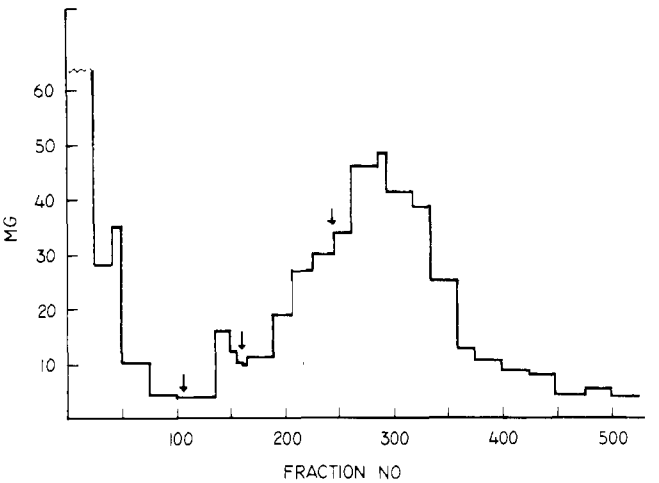

Figure 3. Anion-exchange chromatogram of gymnemate salt extract (15.3 g). Arrows mark the following events: (1) elution with 1 *N* HOAc begun; (2) eluate contains gymnemic acid A_{11} and B_1; (3) eluate free of A_{11}. Fractions after fr 360 contained minor components (Lieberman–Burchard positive) with R_f's intermediate to those of A_{12} and B_1 (tlc, system V).

Conversion of A_{11} to A_{12}. An attempt was made to isolate A_{11} from a forefraction of a large-scale aec preparation of gymnemic acid. The fraction contained no A_{12} (tlc, system V). Conversion to ammonium gymnemate was accomplished by cation-exchange resin with no apparent change in gymnemic acid composition (tlc). In contrast to tlc (silica gel, gypsum binder), dcc (Biosil A, deactivated) converted most of the original A_{11} to A_{12} (tlc, nmr). Weight recovery and tlc of eluted zones indicated that most of the original fraction was neither gymnemic acid nor triterpenoid (negative Lieberman–Burchard reaction).

Spectral Analysis of A_{11} and A_{12}. Infrared spectra (KBr disk) of A_1 (major component A_{12}), A_{11}, and A_{12} were similar to the published spectrum of gymnemic acid A_1 (Figure 4 in Stöcklin *et al.*, 1967). Uv and nmr examination of A_{11} and A_{12} indicated the absence of a tiglate ester moiety in A_{11}.

The uv spectrum of gymnemic acid A_{11} exhibits a broad shoulder at 217 nm (a 14.8) attributable to the conjugated carbonyl system of a tiglate ester [Bueding (1953)]: tiglic acid, uv max 216–17 nm (H_2O, log ϵ 4.0); angelic acid, uv max 217 nm (H_2O, log ϵ 3.7). The nmr spectrum is readily distinguishable from that of A_{12} by a multiplet at δ 6.74 and an intense broad band at δ 1.75 [*cf.*, methyl tiglate: β-H, δ 6.72 (m); β-CH_3, δ 1.75, and α-CH_3, δ 1.80 (complex pattern); Fraser (1960)]. Wulff and Tschesche (1969) have used the olefinic hydrogen multiplet to demonstrate the presence of the tiglate moiety in the saponin aescin. The genin product from enzymatic hydrolysis of gymnemic acid A [Rao (1969): δ 6.18 (m), $CDCl_3$] exhibits similar absorption.

A low-field signal for a formate proton in purified gymnemic acid samples is absent under conditions permitting its detection in butyl formate (δ 1.90, acetone-d_6). The presence of formic acid after saponification of gymnemic acid A (Rao, 1969) may be attributed to artefact formation (chromatography with formic acid solvents) or to lipid impurities in the sample. Power and Tutin (1904) found considerable butyric and formic acid in a saponified petroleum ether extract of an alcoholic fraction (containing gymnemic acid) from "defatted" leaves.

Gel Permeation Chromatography (gpc). Stöcklin *et al.* (1967) reported isolation of an A_1-enriched fraction from gymnemic acid A (A_1–A_4) by gpc on Sephadex G25 with NH_3–NH_4Cl buffer (pH9). Their procedure was applied to a sample of ammonium gymnemate salts containing A_{11} (and B_1) with a Sephadex G25 (Pharmacia) column and NH_4OAc buffer (0.02 N) to avoid alkaline pH. Two colored bands were observed during development, and the void-volume eluate contained A_{11} and B_1 by tlc. All fractions collected thereafter showed similar tlc patterns. The same results were obtained in the absence of buffer salt.

The behavior of gymnemic acid on Sephadex G25 is attributable to its surfactant properties and micellization equilibria (Determan, 1969). The theory of the behavior of micellar solutions in gpc has been treated by Coll (1971). The micellar properties of gymnemic acid have not been emphasized previously. The difficulty in purification may be correlated with solubilization phenomena (Elworthy *et al.*, 1968).

Antisaccharine Activity. In preliminary experiments, the antisweet principle was found associated with the $CHCl_3$–EtOH-soluble fraction. The aqueous residue was not examined for activity or for constituents such as gymnemic acids C and D. The isolation of fractions by aec was initially followed by the psychophysical method of direct magnitude estimation (Bartoshuk *et al.*, 1969; Stevens, 1960). The examination of gymnemic acids A_1, A_{11}, and A_{12} was limited to qualitative assay of the antisweet effect because of the paucity of pure materials. The suppressant effect of 3 ml of 0.03% solutions (*ca.* 3×10^{-4} M) gave a fourfold elevation in threshold for sucrose. The taste of the purified gymnemic acids completely lacked bitterness. For psychophysical studies, even the gymnemate salt extract was sufficiently purified to ascertain previous reports that suppression of bitterness (see references in Stöcklin, 1969b) is a consequence of cross-adaptation to bitter principles in the leaves (Bartoshuk *et al.*, 1969).

Henning (1971) has aptly summarized the theory of chemoreception based on an analogy to regulatory enzymes. The receptor process is envisaged as a binding of the stimulus molecule to a protein receptor site, with a change in conformation by allosteric interaction. Faull *et al.* (1970) have utilized human psychophysical data (Bartoshuk *et al.*, 1969; Meiselman and Halpern, 1970) to show that the mechanism of the action of gymnemic acid is analogous to enzyme inhibition. Their analysis indicated competitive inhibition of judgments to sucrose, and noncompetitive inhibition of sweetness judgments for glycine and alanine. It should be noted that two other plant substances affecting sweetness response, the cationic glycoprotein "miraculin" (sour substances are made to taste sweet: Brouwer *et al.*, 1968; Kurihara and Beidler, 1968), and the cationic protein "monellin" (intense, *long lasting* sweetness), recently characterized by Morris and Cagan (1972), are macromolecules.

In view of the micelle-forming property illustrated by gpc behavior, it might be fruitful to regard gymnemic acid as a macromolecular system (anionic micelle) and the interaction between a membrane-protein receptor and a taste modifier as macromolecule-macromolecule complexing. Studies of the surface activity of gymnemic acids may provide information of use to an understanding of their mode of action *in vivo*.

Further work on the structure of gymnemic acids A_{11} and A_{12} should first ascertain both *quantitatively* and qualitatively what ester groups are present in a manner similar to that pursued by Wulff and Tschesche (1969) in their examination of "aescin."

ACKNOWLEDGMENT

The authors thank Tadeus Reichstein, Walter Stöcklin, and J. E. Sinsheimer for reference samples, and are indebted to L. M. Bartoshuk, F. H. Bissett, Herbert Meiselman, and F. M. Robbins for consultation.

LITERATURE CITED

Bartoshuk, L. M., reported by Pfaffmann, C., in "Olfaction and Taste III," Pfaffmann, C., Ed., Rockefeller University Press, New York, N. Y., 1969, pp 528, 529.
Bartoshuk, L. M., reported by Pfaffmann, C., in "Taste and Smell in Vertebrates," Wolstenholme, G. E. W., Knight, J., Ed., J.&A. Churchill, London, 1970, pp 47–50.
Bartoshuk, L. M., Dateo, G. P., Vandenbelt, D. J., Buttrick, R. L., Long, L., Jr., in "Olfaction and Taste III," Pfaffmann, C., Ed., Rockefeller University Press, New York, N. Y., 1969, p 436.
Belic, I., *Nature (London)* **178**, 538 (1956).
Brouwer, J. N., van der Wel, H., Francke, A., Henning, G. J., *Nature (London)* **220**, 373 (1968).
Bueding, E., *J. Biol. Chem.* **202**, 505 (1953).
Coll, H., *Separ. Sci.* **6**, 207 (1971).
Dateo, G. P., Bartoshuk, L. M., unpublished data, 1967.
Determan, H., "Gel Chromatography," 2nd ed., Springer-Verlag, New York, N. Y., 1969, p 100.
de Zeeuw, R. A., *Progr. Separ. Purif.* **3**, 1 (1970).
Elworthy, P. H., Florence, A. T., Macfarlane, C. B., "Solubilization by Surface-active Agents," Chapman and Hall, London, 1968.
Falconer, *Pharm. J.* **7**, 351 (1848).
Faull, J. R., Halpern, B. P., *Physiol. Behav.* **7**, 903 (1971).
Faull, J. R., Reilly, P. D., Halpern, B. P., presented in part at 41st Annual Meeting of the Eastern Psychological Association, Atlantic City, N. J., April 1970.
Fraser, R. R., *Can. J. Chem.* **38**, 549 (1960).
Henning, G. J., in "Gustation and Olfaction," Ohloff, G., Thomas, A. F., Ed., Academic Press, New York, N. Y., 1971, p 239.
Khastgir, H. N., Sengupta, S. K., Sengupta, P., *J. Indian Chem. Soc.* **35**, 650 (1958).
Kurihara, Y., *Life Sci.* **8**, 537 (1969).
Kurihara, K., Beidler, L. M., *Science* **161**, 1241 (1968).
Loev, B., Goodman, M. M., *Progr. Separ. Purif.* **3**, 73 (1970).
Meiselman, H. L., Halpern, B. P., *Physiol. Behav.* **5**, 945 (1970).
Morris, J. A., Cagan, R. H., *Biochim. Biophys. Acta* **261** 114 (1972).
Pfaffmann, C., in "Handbook of Physiology," Sec 1, Vol. I, American Physiological Society, Washington, D. C., 1959, p 507.
Power, F. B., Tutin, F., *Pharm. J.* **19**, 234 (1904).
Rao, G. S., Ph.D. Thesis, University of Michigan, Ann Arbor, Mich., 1969.
Rao, G. S., Sinsheimer, J. E., *Chem. Commun.* 1681 (1968).
Rao, G. S., Sinsheimer, J. E., *J. Pharm. Sci.* **60**, 190 (1971).
Samuelson, O., *Anal. Chim. Acta* **38**, 163 (1967).

Sinsheimer, J. E., Rao, G. S., McIlhenny, H. M., *J. Pharm. Sci.* **59**, 622 (1970).
Sinsheimer, J. E., Rao, G. S., McIlhenny, H. M., Smith, R. V., Maasab, H. F., Cochran, K. W., *Experientia* **24**, 302 (1968).
Stahl, E., in "Thin-Layer Chromatography," 2nd ed., Stahl, E., Ed., Springer-Verlag, New York, N. Y., 1969, p 86.
Stevens, S. S., *Scand. J. Psychol.* **1**, 27 (1960).
Stöcklin, W., *Helv. Chim. Acta* **52**, 365 (1969a).
Stöcklin, W., *J. Agr. Food Chem.* **17**, 704 (1969b).
Stöcklin, W., Weiss, E., Reichstein, T., *Helv. Chim. Acta* **50**, 474 (1967).
Wagner, J., Schlemmer, W., Hoffmann, H., *Arzneim. Forsch.* **20**, 205 (1970).
Warren, R. M., Pfaffmann, C., *J. Appl. Physiol.* **14**, 40 (1959).
Wulff, G., Tschesche, R., *Tetrahedron* **25**, 415 (1969).
Yackzan, K. S., *Ala. J. Med. Sci.* **3**, 1 (1966).

Received for review August 2, 1972. Accepted May 15, 1973. Presented in part at the Division of Agricultural and Food Chemistry, 161st National Meeting of the American Chemical Society, Los Angeles, Calif., March 1971.

Determination of Organic Acids in Foods by Liquid Chromatography

James K. Palmer* and Dieter M. List[1]

A simple and rapid method for the determination of organic acids in foods by high efficiency anion exchange chromatography is described. Separation of microequivalent amounts of up to 15 of the commonly occurring food acids can be completed in about 75 min. These acids include Krebs cycle acids (citric, isocitric, α-ketoglutaric, succinic, fumaric, malic, and oxalacetic), alicyclic acids (quinic, shikimic), and various other acids (acetic, galacturonic, lactic, malonic, oxalic, and tartaric). Separation of the predominant acids in particular food samples often requires only 20 to 40 min. A special precolumn injection system sharpens the acid peaks and makes possible the analysis of liquid samples (fruit juices, acidulated beverages, etc.) or aqueous extracts with little or no pretreatment. Use of a differential refractometer detector simplifies the quantitative measurements and facilitates collection of effluent samples for confirmation of identity.

A considerable number of organic acids occur in foods and they are often major constituents of plant foods. More than 36 acids are reported to occur in fruits, for example (Hulme, 1970). These acids often influence flavor, stability, and keeping quality and have been proposed as an index of maturity, ripeness, or spoilage in some foods. Certain organic acids (citric, fumaric, lactic, malic, and tartaric) are commonly added to foods as acidulants or flavor modifiers. Organic acids may also be produced during fermentations or other processing operations.

Data on the organic acids in foods are increasingly required by the food industry for quality control to meet legal requirements and as labeling information. Similar data are also required by nutritionists, biochemists, and food scientists during research on the metabolism and function of the organic acids. The "official" chemical methods for organic acids in foods (AOAC, 1970) are simply too time-consuming for most purposes and, in any case, "official" methods are available for only a few acids.

A number of chromatographic methods have been developed for determining organic acids in biological samples. Partition chromatography on silica gel [first utilized for foods by Isherwood (1946)] and anion exchange chromatography [as originally proposed by Busch *et al.* (1952)] have emerged as the methods of choice, since other methods such as gas chromatography have significant disadvantages (Stahl *et al.*, 1972). Numerous applications or adaptations of these two techniques have been reported (Bengtsson and Samuelson, 1969, 1971; Kesner and Muntwyler, 1969; Palmer, 1955; Palmer and Wyman, 1965; Von Korff, 1969). However, these are slow and cumbersome procedures, at least in relation to recent developments in high efficiency liquid chromatography (Kirkland, 1971). Separation of nanoequivalent quantities of organic acids has been accomplished in high efficiency systems by both partition (Stahl *et al.*, 1972) and anion exchange chromatography (Bengtsson and Samuelson, 1971). These procedures apparently have adequate resolution only at the nanoequivalent level, thus limiting the sample size and requiring the use of rather complicated photometric detection systems. The ion exchange system also requires up to 16 hr to separate the commonly occurring organic acids. For most food analyses (and indeed for many other biological samples) simplicity, speed, reliability, and ease of sample preparation are more important criteria than ultimate sensitivity.

The general approach in this study has been to adapt the anion exchange procedure of Palmer (1955) to a high efficiency system, including the use of fine, spherical resins and a differential refractometer for detection. The resulting method combines simplicity and minimum sample preparation with adequate speed, sensitivity, and precision for food analysis. The method appears to have application to the determination of organic acids in other biological systems.

EXPERIMENTAL SECTION

Liquid Chromatography. Following is a list of instruments, materials and conditions used in the liquid chromatographic operation. Waters Associates ALC-100, differential refractometer detector, sensitivity 1×10^{-7} refractive index units; attenuation 2–16. Texas Instruments Servo/riter II recorder, 50 mV full scale, chart speed 0.15 in./min. Columns: precolumn, length 3.6 cm; separation columns, length 75–90 cm; all columns of 316 stainless steel, ¼ or ⅜ in. o.d. by 0.028-in. wall thickness. Column

Department of Nutrition & Food Science, MIT, Cambridge, Massachusetts 02139.
[1] Permanent address: Institute of Fruit and Vegetable Technology, Technical University of Berlin, Berlin, Germany.