Compounds other than phenothiazines were also investigated for their ability to block protoveratrine induced emesis. Three potent antiemetic agents, haloperidol, meclizine, and trimethobenzamide (12, 13) were found to be without effect. Similarly, atropine, nicotinic acid, and P-275, which have not been reported to be antiemetic, were ineffective. Furthermore, chlorphenoxamine and reserpine did not consistently block PAB emesis. Other workers (6, 14) have reported that parasympatholytics (atropine, scopolamine, and methantheline), a ganglionic blocker (tetraethylammonium), a sympathomimetic (ephedrine), an antihistaminic (dimenhydrinate) and reserpine failed to prevent veratrum emesis in dogs.

Until more data are forthcoming on the mechanism of the emetic action of protoveratrine A, the inability of potent antiemetics to prevent the effect will remain obscure.

SUMMARY

Protoveratrine A is equally potent as an emetic agent by the intravenous and oral routes. On the other hand, protoveratrine B is only one-fifth as potent orally as by the i.v. route.

Neither the rate of injection nor the volume of solution containing the emetic dose of protoveratrine A is important with respect to modifying the side effects which usually accompany the intravenous injection of the drug.

Acute tolerance does not develop to the emetic action of protoveratrine A. Six out of seven dogs which responded to a challenging dose also vomited when the dose was repeated after an interval of 15, 30, 40, 120, or 135 minutes. Tolerance developed to the convulsive-like effects.

None of the drugs tested in this study were effective in preventing protoveratrine induced emesis in dogs.

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Gas Chromatography of Esters of Plant Acids and Their Identification in Plant Materials

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The gas chromatographic retention data of methyl and ethyl esters of a number of common plant acids are reported. Special emphasis is placed on the acids of the Krebs cycle. Of four liquid phases used, Apiezon L appears to be most generally applicable and the only one which permits satisfactory separation of fumarate and succinate. The esters of citric acid and isocitric acid (lactone) are readily separated on all four columns. The method has been used for identification of the acids in various plant materials. It is sensitive and selective and should be valuable for biochemical studies concerning the production and function of organic acids in plants.

PLANTS CONTAIN a large number of organic acids, some of which may be characteristic of certain plant families. Others, such as a group of di and tricarboxylic acids, are found more widely distributed throughout the vegetable kingdom and are usually referred to simply as the plant acids.

In 1937, Krebs and Johnson (1) showed that these acids play a central role in cellular respiration, and the series of reactions which are involved is known as the Krebs cycle. Some of the acids of the Krebs cycle, such as succinic, fumaric, malic, and citric acids, are found in appreciable concentrations in practically every type of plant material. Most of the others are present only in small amounts and require sensitive and specific methods for their detection. The keto acids, particularly oxaloacetic acid, are unstable and may easily be lost during the isolation procedures commonly used for plant acids and may thus escape detection. The enzymes

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required for the various steps of the Krebs cycle have also been identified in higher plants (2).

Certain plant acids belonging to the Krebs cycle tend to accumulate in special plant families. Isocitric acid, for example, is found in large concentration in succulents of the *Crassulaceae* family; before its chemistry and biological significance was known, it was commonly referred to as crassulacean malic acid (3). Aconitic acid has been found in many plants, but is present in large amounts in *Aconitum septentrionale* L. (4). Such an accumulation of individual plant acids indicates that there is a partial block of a particular step in the cycle due perhaps to a low enzyme concentration or the presence of an enzyme inhibitor (2).

TABLE I.—RELATIVE RETENTION TIMES OF METHYL ESTERS OF PLANT ACIDS

	Stationary Liquid		
Ester	SE-30[a]	Apiezon L	Craig Ester[b]
Oxalate	0.09	0.14	0.19
Malonate	0.20	0.22	0.23
Succinate	0.39	0.43	0.29
Fumarate	0.36	0.48	0.28
Oxaloacetate	0.66	1.04[c]	...
Malate	1.00	1.00	1.00
α-Ketoglutarate	1.85	1.60	1.12
Tartrate	3.15	2.55	2.80
Aconitate	10.42	4.56	2.06
Citrate	15.73	6.08	4.16
Lactoisocitrate	21.20	10.33	11.50
Temp., °C.	55	122	182
Inlet pressure p.s.i.	20	30	25
Outlet flow rate, ml./min.	74	...	...
Carrier gas	Argon	Nitrogen	Nitrogen
Support, mesh	60–80	60–80	60–80
Liquid phase, % w/w	4	15	10
Malate time, min.	6.1	5.9	6.5

[a] Methyl silicone polymer, General Electric Co. [b] Butanediol succinate. [c] The sample appeared to decompose on the column; no well defined peak was obtained.

Three dicarboxylic acids commonly found in plants are not components of the Krebs cycle—oxalic, malonic, and tartaric acid. It has been postulated that oxalic acid may be formed from glycolic acid *via* glyoxylic acid (2). One might also speculate concerning the function of malonic acid which is a highly specific inhibitor of succinic dehydrogenase.

For biochemical studies of the plant acids, it is imperative to have analytical methods which are suitable for qualitative and quantitative determination of micro quantities. The most common method for isolation of plant acids is based on precipitation with lead acetate or lead subacetate, followed by liberation of the free acids by hydrogen sulfide. The individual acids may then be identified and determined by fractional precipitation or by fractional distillation of their esters. However, neither of these methods produces complete separation nor permits determination of an acid present in small quantities. The ester-hydrazide method of Franzen, *et al.* (5, 6), represents a considerable advancement. More recent work (7) has made use of fairly specific micro methods based on chemical and enzymatic reactions. Ion-exchange resins and paper partition chromatography have in many instances given excellent results (8, 9). However, the separation of individual acids is often not sufficient to identify and determine small amounts of certain acids in the presence of large amounts of others.

It would seem that gas-liquid chromatography might offer considerable advantages over the methods presently used. It is extremely rapid and sensitive, highly selective, and lends itself equally well to qualitative and quantitative analysis. Furthermore, it permits determination of trace components of a mixture. Gas chromatography was developed by Martin and James

TABLE II.—RELATIVE RETENTION TIMES OF ETHYL ESTERS OF PLANT ACIDS

	Stationary Liquid			
Ester	SE-30	Apiezon L	Igepal[a]	Craig Ester
Oxalate	0.18	0.21	0.13	0.19
Malonate	0.26	0.30	0.20	0.26
Maleate	0.49	0.54	0.35	0.39
Succinate	0.54	0.57	0.26	0.33
Fumarate	0.56	0.67	0.26	0.32
Malate	1.00	1.00	1.00	1.00
α-Ketoglutarate	1.85[b]	1.42[b]	...	1.22[b]
Tartrate	2.03	1.86	...	2.90
Aconitate	10.61	5.46	...	2.15
Citrate	12.72	6.35	...	4.25
Lactoisocitrate	17.08	7.07	...	10.17
Temp., °C.	80	150	130	182
Inlet pressure, p.s.i.	20	30	20	25
Outlet flow rate, ml./min.	67	...	100	...
Carrier gas	Argon	Nitrogen	Argon	Nitrogen
Support, mesh	60–80	60–80	60–80	60–80
Liquid phase, % w/w	4	15	5	10
Malate, min.	3.6	4.3	6.8	4.7

[a] Nonylphenoxypolyethyleneoxy—ethanol. [b] Major peak.

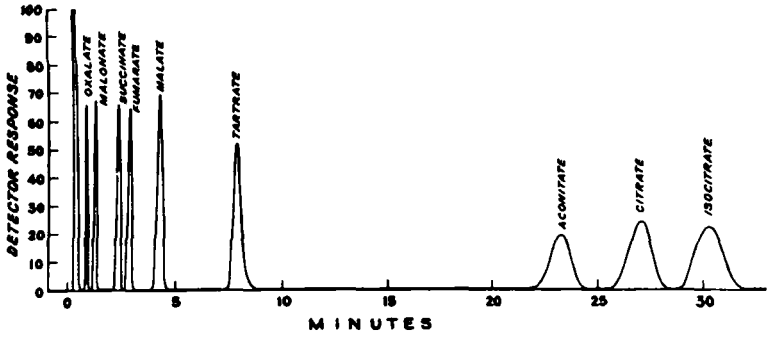

Fig. 1.—Gas chromatogram of the ethyl esters of nine plant acids on Apiezon L. The experimental conditions are shown in Table I.

(10) in 1952 as a tool for studying fatty acids. Since that time, it has become so widely used that it has practically displaced all other methods for routine analysis of fats and oils and for biochemical research concerning fatty acids. Much less work has been done with gas chromatography of di and tricarboxylic acids. James, *et al.* (11), determined the structure of unsaturated fatty acids by oxidative degradation followed by esterification and gas chromatography of the dicarboxylic acids produced. Nowakowska, *et al.* (12), and Miwa, *et al.* (13), used gas chromatography to separate and identify the dicarboxylic acids formed during oxidation of fats and oils. The dicarboxylic acids present in cigarette smoke were identified and determined quantitatively by Quin and Hobbs (14). In his book on gas chromatography, Bayer (15) refers to unpublished work dealing with gas chromatographic separation of methyl esters of certain di and tricarboxylic acids belonging to the Krebs cycle.

EXPERIMENTAL

Esterification of Plant Acids.—Ethyl esters were produced by refluxing the dried acids with anhydrous ethanol containing 5% of hydrogen chloride. With small samples of acids (10 to 20 mg.), the esterification appeared to be complete in about 2 hours. The reaction mixture was evaporated almost to dryness in a rotating vacuum evaporator. The residue was transferred to a small separator, 5 ml. of water was added, and the ester was extracted twice with 10 ml. of ether. The combined ether extracts were washed with a little sodium bicarbonate solution, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure at room temperature.

The methyl esters were synthesized by three different methods, all of which gave satisfactory results with most acids. The method which appeared to be most generally applicable was esterification with diazomethane. The microscale procedure described by Schlenk and Gellerman (16) produced methyl esters in good yield from most of the acids. Depending on the nature of the acid, the esterification was complete in from 10 to 30 minutes. Side reactions may occur with special acids unless the reagent concentration is carefully controlled. Keto acids gave rise to by-products by reaction between

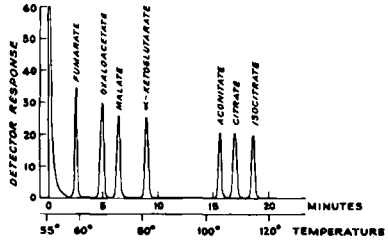

Fig. 2.—Temperature programmed gas chromatogram of methyl esters of plant acids on silicone rubber SE-30, 4%.

diazomethane and the keto group. These acids were best esterfied with methanol catalyzed by boron trifluoride (17). The acid (10 to 100 mg.) was dissolved in 3 ml. of BF_3-methanol reagent in a test tube, and the solution was heated gently on a steam bath for 2 minutes, then cooled under running water and transferred to a small separator with the aid of about 5 ml. of water. The ester was extracted twice with 10 ml. of ether. The combined ether extracts were dried over a little anhydrous sodium sulfate, filtered, and evaporated to dryness at room temperature under reduced pressure. This method appeared to be effective with most of the acids studied, but gave low yield with tartaric acid.

The third method used for production of the methyl esters was similar to the procedure described for the ethyl esters. The keto acids showed considerable decomposition by this method, evidenced by the appearance of several peaks on the gas chromatogram. However, the other acids gave methyl esters in good yield.

Isocitric acid is not known as the free tricarboxylic acid in a crystalline state. When evaporated and dried, the acid loses water and is converted to the lactone (3). Isocitric acid lactone and its barium salt are commercially available. On esterification, both compounds gave esters which had the same retention times as the corresponding authentic diethyl and dimethyl esters of lactoisocitric acid.[1]

Isolation of Acids from Plant Materials.—Ten grams of finely ground plant materials was extracted twice with 100 ml. of warm ethanol each time. The combined extracts were evaporated to dryness under reduced pressure, the residue was dissolved in about 25 ml. of water, and filtered. The filtrate was heated to about 60°, and a saturated solution of lead acetate was added until no further precipitation took place. Lead subacetate solution was then

[1] We are indebted to Professor A. Nordal of Oslo University for supplying us with these authentic esters.

added in the same way. The total lead precipitate was filtered off, washed with water, suspended in 50 ml. of 50% ethanol, and the free acids were regenerated with hydrogen sulfide. The lead sulfide was removed by filtration, and the filtrate evaporated to dryness in a rotating vacuum evaporator at a low temperature. The last traces of moisture were removed by addition of absolute ethanol and evaporation to dryness. This procedure was repeated twice. The acid residue was esterified as described above.

Gas Chromatography of Esters of Plant Acids.— Two gas chromatographs were used for the work reported here. One was a Barber-Colman model 15 gas chromatograph equipped with an argon ionization detector (50 μc. of Ra-226). The columns for this instrument were glass U-tubes, 4 ft. long and 4 mm. in inside diameter. The other instrument was a Wilkins HY-FI gas chromatograph having a hydrogen flame ionization detector and stainless steel columns, 6 ft. long and ⅛ in. in outside diameter. Gas-Chrom P was used as the solid support. It was washed with concentrated hydrochloric acid, methanolic potassium hydroxide, dried, and treated with hexamethyldisilazane (18). The dried and siliconized support was coated with the liquid phase as described by Horning, *et al.* (19). When the stationary phase was nonpolar, such as Apiezon or silicone rubber SE-30, a coating of 0.1% of polyethylene glycol 9000 was applied prior to the main liquid phase (18, 20). This did not seem to alter the retention data, but markedly reduced tailing of the polar hydroxy esters. The ester sample was injected into the flash heater, maintained at 175°, with a Hamilton microsyringe. The usual sample was 0.5 μl. of an acetone solution containing 2 to 10 mcg. of each ester. If the flash heater temperature was much above 175°, certain decomposition reactions could sometimes be observed. Malic acid esters, for example, under such conditions produced three peaks, due to partial dehydration to maleate and fumarate.

RESULTS AND DISCUSSION

The gas chromatographic retention data of the esters of 12 di and tricarboxylic acids are given in Tables I and II. Maleate is included because of its structural relationship to fumarate, although the acid has never been isolated from plant materials. A typical gas chromatogram of esterified plant acids is illustrated in Fig. 1. Temperature programing is useful in speeding up the elution of aconitate, citrate, and isocitrate and producing sharper peaks. Figure 2 represents a temperature programed gas chromatogram on silicone rubber SE-30.

It is apparent from Tables I and II that the greatest number of esters could be separated on Apiezon L. This stationary phase was, furthermore, the only one that produced satisfactory separation of succinate and fumarate. The geometric isomers, maleate and fumarate, were separated as the ethyl esters on all four columns. Maleate and succinate, however, could only be separated on polar columns. Citrate and lactoisocitrate showed great differences in retention times.

The acetone solutions of the esters are unstable and should be gas chromatographed without much delay. The esters of the keto acids and the hydroxy-acids are particularly subject to decomposition in solution. After a day or two, several gas chromatographic peaks were produced indicating substances which were not present in the original solution.

The plant acids were isolated from *Celtis occidentalis, Urticaceae*, as described above. The following acids were identified: oxalic, malonic, fumaric, succinic, malic, citric, and isocitric.

The dried roots of *Angelica archangelica* L. subsp. *norvegica, Umbelliferae*, contained oxalic, malonic, fumaric, succinic, malic, aconitic, and citric acid. This corresponds to the acids identified in this plant by fractional distillation of the esters, followed by hydrolysis and paper chromatography of the liberated acids (21).

A fraction of ethyl esters from *Sempervivum tectorum* L., *Crassulaceae*,[2] showed the presence of succinic, malic, citric, and isocitric acids.

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[2] The ethyl ester fraction was kindly supplied by Professor A. Nordal.