above structural observations with some of the properties found for this compound.

(*a*) The high optical rotation and its temperature dependence might find its explanation in that the electronic state about the disulfide linkage and the conformation of the sugar moieties may hinder free rotation of the two nucleoside residues about the S—S bond.

(*b*) The facile reducibility of the disulfide linkage for this compound appears to be attributable to the valence state of the disulfide bond.

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Essential Oils and Their Constituents XXXIV

Isolation of Khusenic Acid and Isokhusenic Acid from Oil of Vetiver and Some Observations Concerning Their Structures

By ISHWAR C. NIGAM and HISASHI KOMAE

Khusenic acid and isokhusenic acid, two new tricyclic sesquiterpene acids, were isolated from oil of vetiver (*Vetiveria zizanioides*, Linn). The physicochemical properties of the acids and their methyl esters were determined. Infrared, NMR, and mass spectral data as well as the results of dehydrogenation suggest that the two acids differ in the location of the double bond. Khusenic acid and methyl khusenate possess a terminal methylene group which rearranges in the presence of mineral acid to a tetrasubstituted double bond leading to the formation of isokhusenic acid and methyl isokhusenate, respectively. Dehydrogenation of the acids as well as their esters failed to yield any aromatic products, the main reaction being decarboxylation of the compounds to a C_{14} hydrocarbon.

OIL OF VETIVER, an important essential oil used by the perfume and cosmetic industries, is obtained by steam distillation of the roots of *Vetiveria zizanioides* (*Gramineae*). Chemical examination of the oil has been carried out by many investigators for almost a century. Most of this work concerned its ketone, alcohol, and hydrocarbon fractions. The acids present in the oil have so far received only limited attention (1–4). The present report describes the isolation and characterization of the major acidic constituent as well as of a closely related isomer.

It is suggested that the major acidic constituent be named khusenic acid (3) rather than vetivenic acid (5) as the latter nomenclature may erroneously imply structural relationship with vetivane. The isomeric compound will accordingly be named isokhusenic acid.

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Angola oil (acid value: 29.1) gave a higher recovery of acids than reunion oil (acid value: 6.3), and was, therefore, used in the present investigations. Since gas chromatographic analysis of carboxylic acids is best carried out on the esters, the acids were isolated as methyl esters using Amberlite IRA-400 ion-exchange resin (6). This method was superior to that involving extraction with $NaHCO_3$, hydrolysis of the salt with HCl, and subsequent methylation, as it yielded the esters directly in a higher yield and the isolated fraction was lighter in color.

The gas chromatograms of the ester fractions obtained by either method exhibited essentially the same peaks but the relative proportions of the constituents appearing at 16.2 min. and 13.1 min. were reversed. It appeared that isolation of the acids by the $NaHCO_3$ method, particularly regeneration of the acids from the sodium salts by treatment with hydrochloric acid, brought about isomerization of the main component, khusenic acid to isokhusenic acid. This was confirmed by treating methyl khusenate with aqueous methanolic HCl, which yielded methyl isokhusenate as the major product of reaction (see Fig. 1 and Table I).

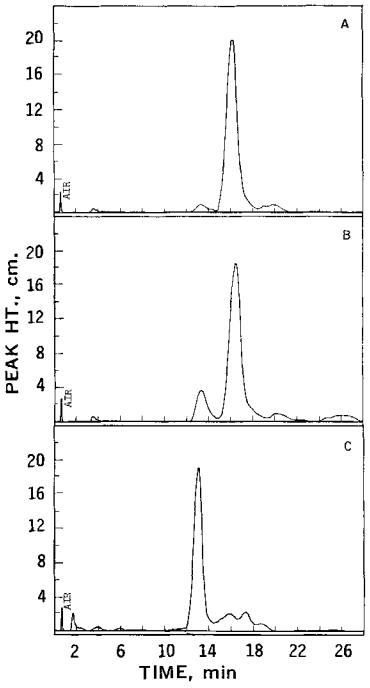


Fig. 1—Gas chromatograms of methyl esters of free acids present in oil of vetiver: A, resin treatment and methylation at room temperature; B, resin treatment and methylation under reflux; C, isomerization of A with HCl. Column: Reoplex 400, operated at 220°. Carrier gas: helium, 75 ml./min.

Analytical samples of methyl khusenate and methyl isokhusenate were obtained by preparative gas chromatography of the crude ester fraction and its isomerization product, respectively. Elemental analysis of methyl isokhusenate and molecular weights of both esters determined by mass spectrometry agreed with the molecular formula $C_{16}H_{24}O_2$. Hence, the corresponding acids are $C_{15}H_{22}O_2$.

Saponification of methyl isokhusenate yielded isokhusenic acid, which on purification *via* its cyclohexylamine salt (m.p. 131–133°) was obtained as a crystalline solid (m.p. 80–81°). For the preparation of pure khusenic acid, the crude acid obtained *via* $NaHCO_3$ extraction was converted into the cyclohexylamine salt (m.p. 146–148°), and the derivative decomposed with dilute hydrochloric acid. The acid so obtained was a liquid. Gas chromatographic analysis (Reoplex 400 column) showed it to be a mixture of two substances, which were isolated by preparative gas chromatography. The faster eluting one was a solid (m.p. 80–81°), the other a liquid. On the basis of infrared and NMR spectral data, the compounds were identified as isokhusenic acid and khusenic acid, respectively.

Hydrogenation of methyl khusenate and methyl isokhusenate confirmed the presence of one double bond in each molecule and hence the tricyclic character of the esters as well as the corresponding acids (2–4). The nature of unsaturation in both compounds was revealed by infrared and NMR spectral analysis as well as ozonolysis.

TABLE I—GAS CHROMATOGRAPHIC ANALYSIS OF METHYL ESTERS OF FREE ACIDS ISOLATED BY DIFFERENT METHODS

No.	Origin of Oil	Method of Obtaining Esters	—% of Main Components[a]— Methyl Khusenate	Methyl Isokhusenate
1	Reunion	$NaHCO_3$ treatment and methylation of the regenerated acid	13	60
2	Reunion	Resin treatment and methylation at room temperature	66	11
3	Angola	Same as in 2	88	5
4	Angola	Resin treatment and methylation under reflux	69	14
5	Angola	Sample from 3 isomerized with HCl	14	73

[a] The balance of 100% represents unidentified minor constituents.

The infrared spectra of the two acids and their esters are shown in Fig. 2. Khusenic acid exhibited infrared absorption bands at 3080, 1635, and 888 cm.$^{-1}$, characteristic of an unsymmetrical disubstituted double bond (>C=CH_2) (7). The corresponding peaks in the spectrum of methyl khusenate appeared at 3085, 1637, and 890 cm.$^{-1}$. The infrared spectra of isokhusenic acid and its methyl ester lacked characteristic olefinic absorption bands, thereby indicating the tetrasubstituted nature of the double bond in both these compounds.

The NMR spectra of the two acids and their methyl esters supported the infrared assignments and in addition suggested the presence of two more methyl groups in these compounds. Thus khusenic acid and methyl khusenate showed peaks for the >C=CH_2 group at 4.75 and 4.61 δ and at 4.77 and 4.63 δ, respectively. Isokhusenic acid and methyl isokhusenate did not give any signal attributable to an olefinic proton. These compounds, on the other hand, exhibited peaks at 1.47 and 1.46 δ, respectively, which could be assigned to a CH_3 group attached to an olefinic carbon atom. In addition, all four compounds gave peaks indicative of the presence of two paraffinic methyl groups. For isokhusenic acid and its ester, these groups possessed identical chemical shifts and generated a single peak. The positions and assignments of characteristic peaks observed in the NMR spectra of these compounds are summarized in Table II.

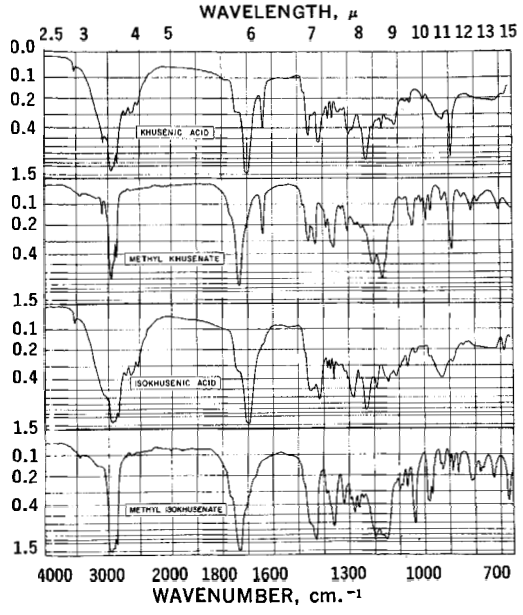
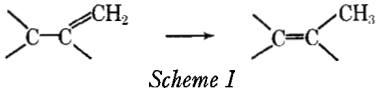


Fig. 2—Infrared spectra of khusenic acid, isokhusenic acid, and their methyl esters. The ordinate is labeled absorbance.

On ozonolysis methyl khusenate lost one carbon atom and yielded a monoketo ester ($C_{15}H_{22}O_3$). The infrared spectrum of the product exhibited carbonyl bands at 1708 and 1728 cm.$^{-1}$ for the keto group and the ester group, respectively. The observation confirmed the presence of an exocyclic double bond in methyl khusenate and hence, in the corresponding acid.

On the basis of the above data it would appear that the acid-catalyzed isomerization of khusenic acid and its methyl ester involves the rearrangement of the unsaturated linkage shown in Scheme I. Close resemblance between the



Scheme I

infrared spectra of the two acids and their methyl esters in the fingerprint region and the generation of peaks at the same positions in their mass spectra (Fig. 3) suggests that the isomerization involves only the double bond and does not affect any other part of the molecule. This deduction was further supported by the results of dehydrogenation experiments, in which the acid as well as their esters yielded the same major reaction product.

In none of these experiments was any aromatic or azulenic compound obtained. A hydrocarbon ($C_{14}H_{22}$) was always the main reaction product. The infrared spectrum of this hydrocarbon did not exhibit any absorption characteristic of aromatic or olefinic groups. The NMR spectrum indicated the presence of three methyl groups, one of them attached to an olefinic carbon atom

TABLE II—NMR SPECTRAL DATA FOR KHUSENIC ACID, ISOKHUSENIC ACID, AND THEIR DERIVATIVES

Compd.	Positions (δ) of Characteristic Peaks and Assignments: =CH_2	$COOCH_3$	=C—CH_3	C—CH_3
Khusenic acid	4.75, 4.61 (2H)	...	...	1.09 (3H)
				1.03 (3H)
Isokhusenic acid	...	...	1.47 (3H)	1.00 (6H)
Methyl khusenate	4.77, 4.63 (2H)	3.67 (3H)	...	1.08 (3H)
				1.05 (3H)
Methyl isokhusenate	...	3.64 (3H)	1.46 (3H)	1.00 (6H)
C_{14} Hydrocarbon obtained by dehydrogenation	...	...	1.47 (3H)	1.02 (3H)
				1.98 (3H)

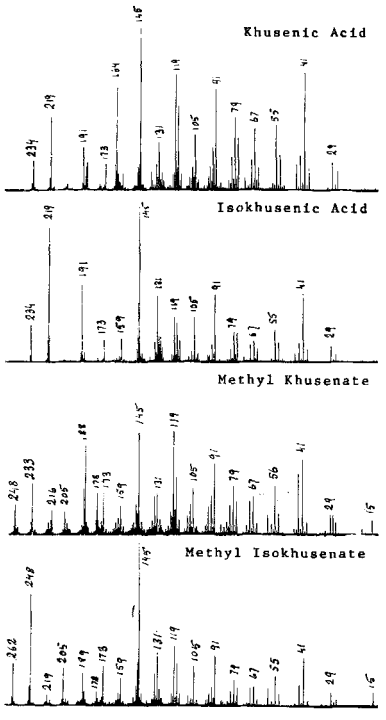


Fig. 3—Mass spectra of khusenic acid, isokhusenic acid, and their methyl esters.

(see Table II). These data suggested that in the case of isokhusenic acid and its methyl ester the reaction involved primarily loss of the carboxyl or the ester group, respectively. In the case of khusenic acid and its methyl ester it involved, in addition, rearrangement of the olefinic methylene group to a tetrasubstituted double bond, a reaction analogous to the isomerization of these compounds observed under the influence of acid.

The experimental data presented describe, for the first time, standardized procedures for the preparation of khusenic acid, isokhusenic acid, and their methyl esters, and yield some preliminary information regarding the structures of these substances. Isokhusenic acid and its methyl ester are crystalline solids and can, therefore, be readily characterized. They may also serve for the identification of khusenic acid and its methyl ester from which the former may be easily prepared *via* isomerization. The spectral and gas chromatographic data should prove of value as additional criteria of identity. Although both acids yield solid cyclohexylamine salts, these compounds were not found to be sufficiently stable for use as characteristic derivatives.

EXPERIMENTAL

All melting points and boiling points are uncorrected. Microanalyses were carried out gas chromatographically employing an F&M, C, H, and N analyzer model 185. Infrared spectra were recorded on a Perkin-Elmer model 221 spectrophotometer and NMR spectra measured in $CDCl_3$ solution employing TMS as internal reference using a Varian A-60A spectrometer equipped with a Varian C-1024 time averaging computer (CAT). Mass spectra were obtained with a Hitachi-Perkin-Elmer RMU-6D spectrometer. Measurements of optical rotations were carried out in $CHCl_3$ solution using a Rudolph model 200 photoelectric polarimeter. Gas chromatographic analyses were carried out on two instruments: (*a*) Burrell Kromo-Tog K-2 equipped with a 20% Reoplex 400 column prepared as previously described, the carrier gas being helium (75 ml./min.) (8); (*b*) aerograph Hi-Fi 600-C equipped with a stainless steel column (outside diameter $^1/_8$ in., length 5 ft.) containing 3% silicone rubber SE-30 on silanized 60 to 80 mesh Chromosorb W prepared according to the method of Horning *et al.* (9, 10), the carrier gas being nitrogen (16 ml./min.).

Isolation of Acids from Reunion Vetiver Oil by Extraction with $NaHCO_3$—The oil (175 Gm.) was dissolved in hexane (350 ml.) and shaken with five 175-ml. portions of 10% $NaHCO_3$ solution. The aqueous extract was washed with water (100 ml.), acidified with 1 *N* HCl solution, and the liberated acid was extracted with five 200-ml. portions of ether. The ether extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated. Yield of crude acids: 1.86 Gm. (1.1%).

Preparation of Methyl Esters—A portion of the crude acids (840 mg.) was dissolved in the methylating reagent (20 ml.) prepared by adding acetyl chloride dropwise to ten times its volume of cold absolute methanol. The mixture was left overnight, then diluted with water (80 ml.), and extracted five times with 20-ml. portions of ether. The ether extract was freed from HCl by repeated washings with water, dried over anhydrous Na_2SO_4, and evaporated. Yield of methyl esters: 572 mg. (68.1%).

Isolation of Free Acids as Methyl Esters from Reunion Vetiver Oil by Treatment with Ion-Exchange Resin—Oil of vetiver (2 Gm.) was dissolved in hexane (25 ml.), and the mixture was stirred for 5 min. with Amberlite IRA-400 (10 Gm.) converted into the OH form by pretreatment with alkali. Following decantation the resin was washed with 25-ml. portions of hexane until evaporation of the washings did not yield an oily residue. The oil-free resin was allowed to react with the above described methylating reagent (25 ml.) for 30 min. with frequent shakings. The supernatant liquid was decanted and the resin washed twice with 10 ml. of the methylating reagent. The supernatant liquid and the washings were combined, and the methyl esters were isolated from the reaction mixture in accordance with the procedure described. Yield of esters: 118 mg. (5.9%).

Isolation of Free Acids as Methyl Esters from Angola Vetiver Oil by Treatment with Ion-Exchange Resin—A sample of the oil (5.05 Gm.), was allowed to react with Amberlite IRA-400 resin (40 Gm.), as described above. The acid-bearing resin was divided in two equal parts. One part was methylated (50 ml. reagent) for 30 min. as previously described and the other part was refluxed with the same volume of reagent for the same length of time. The

methyl esters were recovered from each reaction mixture as described before. Yields of the ester fractions: obtained at room temperature, 661 mg. (26.2%); under reflux, 581 mg. (23.0%).

Acid-Catalyzed Isomerization of Esters Obtained by Resin Treatment—A sample of the ester (104 mg.) obtained from Angola oil by methylation at room temperature was dissolved in a mixture of methanol (9 ml.) and concentrated HCl (1 ml.), and refluxed for 2 hr. Following conventional processing the reaction mixture gave the isomerized product, yield: 101 mg. (97.1%).

Gas Chromatography of Ester Fractions—The esters obtained in accordance with the procedures described above were gas chromatographed on Reoplex 400 and SE-30 columns, operated at 220° and 180°, respectively. All samples exhibited two main peaks corresponding to methyl khusenate and methyl isokhusenate. The relative retention times of the two constituents on the Reoplex 400 column (reference standard: 1,2-dimethylnaphthalene) were 1.59 and 1.31, and on the SE-30 column (reference standard: phenanthrene) 0.85 and 0.77, respectively. The relative proportions of methyl khusenate and methyl isokhusenate in the different samples are recorded in Table I.

Preparation and Properties of Methyl Khusenate and Methyl Isokhusenate—The crude ester fractions were subjected to fractional distillation under reduced pressure—methyl khusenate fraction, yield 87%, purity by GLC 92%; methyl isokhusenate fraction, yield 90%, purity by GLC 95%. Methyl isokhusenate was further purified by recrystallization from ethanol, and an analytical sample of methyl khusenate was obtained by preparative gas chromatography on the Reoplex 400 column. The properties of the two esters are described below.

Methyl Khusenate—$b_{0.7}$ 130°; $[\alpha]_D$ +43.5° (c 3.54); n_D^{26} 1.5058; mol. wt. (mass spectrum) 248.

Anal.—Calcd. for $C_{16}H_{24}O_2$: C, 77.37; H, 9.74. Found: C, 77.60; H, 9.79.

Infrared absorption bands (liquid film) at 3085, 1730, 1637, 1457, 1430, 1385, 1365, 1351, 1303, 1200, 1165, 1045, 991, 973, 930, 890, 850, 815, 790, and 707 cm.$^{-1}$; NMR peaks at 4.63, 4.77, 3.67, 1.08, and 1.05 δ.

Methyl Isokhusenate—Melting point 51°; $b_{0.6}$ 125°; $[\alpha]_D$ +46.8° (c 1.64); mol. wt. (mass spectrum) 248.

Anal.—Calcd. for $C_{16}H_{24}O_2$: C, 77.37; H, 9.74. Found: C, 77.60; H, 9.66.

Infrared absorption bands (melt film) at 1732, 1452, 1432, 1387, 1360, 1320, 1293, 1278, 1261, 1243, 1199, 1190, 1168, 1153, 1098, 1087, 1069, 1040, 981, 968, 937, 929, 902, 888, 867, 815, 780, 770, and 728 cm.$^{-1}$; NMR peaks at 3.64, 1.46, and 1.00 δ.

Preparation of Isokhusenic Acid from Methyl Isokhusenate—Methyl isokhusenate (203.9 mg.) was refluxed with 1 *N* methanolic KOH (5 ml.) for 3 hr. The reaction mixture was acidified (pH 3) with 1 *N* HCl and extracted with three 20-ml. portions of ether. The ether extract on conventional processing yielded crude isokhusenic acid (160.4 mg.). Reaction of the crude acid with cyclohexylamine (0.2 ml.) in acetone (2 ml.) yielded the cyclohexylamine salt recrystallized from the same solvent (m.p. 131–133°). Isokhusenic acid was regenerated from the salt by treatment with 1 *N* HCl (2 ml.). The liberated acid was extracted with ether (3 × 2 ml.); the extract was washed with water, dried over Na_2SO_4, and evaporated. The residue, on recrystallization from aqueous ethanol, yielded pure isokhusenic acid (26.4 mg.), m.p. 80–81°; $[\alpha]_D$ +27.9° (c 2.78).

Anal.—Calcd. for $C_{15}H_{22}O_2$: C, 76.88; H, 9.46. Found: C, 77.32; H, 9.61.

Infrared absorption bands (CCl_4 solution) at 3520, 3000–2400 (broad band), 1700, 1453, 1418, 1384, 1372, 1359, 1281, 1231, 1191, 1144, 1068, and 938–928 cm.$^{-1}$; NMR peaks at 1.47 and 1.00 δ.

Isolation of Khusenic and Isokhusenic Acids from Crude Acid Fraction—The dark viscous acid fraction (1.0 Gm.) obtained from Reunion vetiver oil following treatment with $NaHCO_3$ was allowed to react with cyclohexylamine (1.0 ml.) in acetone solution (5 ml.). The cyclohexylamine salt separated as a solid which was recrystallized from acetone (m.p. 146–148°). On treatment with 1 *N* HCl (3 ml.) it yielded an oil (650 mg.), which by gas chromatographic analysis was found to be composed of khusenic acid (52%) and isokhusenic acid (48%) (retention times: 55.0 min. and 44.5 min., respectively; column: Reoplex 400; temperature 220°). Each of the two acids were collected separately by gas chromatography. The I.R. spectrum and melting point of isokhusenic acid were identical with those of the sample prepared from methyl isokhusenate. Khusenic acid had the following properties: $b_{0.5}$ 158°; $[\alpha]_D$ +17.2 (c 4.39); n_D^{26} 1.5198.

Anal.—Calcd. for $C_{15}H_{22}O_2$: C, 76.88; H, 9.46. Found: C, 77.34; H, 9.55.

Infrared absorption bands (CCl_4 solution) at 3520, 3080, 1700, 1635, 1457, 1415, 1378, 1362, 1335, 1297, 1225, 1165, 1114, 943–928, and 888 cm.$^{-1}$; NMR peaks at 4.75, 4.61, 1.09, and 1.03 δ.

Hydrogenation of the Methyl Esters—Methyl khusenate and methyl isokhusenate were hydrogenated in glacial acetic acid solutions employing Adam's platinum oxide catalyst. The volume of hydrogen absorbed by 105 mg. of methyl khusenate at 27° and 754 mm. pressure was 13 ml. (1.2 moles of H_2 per mole of ester). Hydrogen uptake by 100 mg. of methyl isokhusenate under the same conditions was 10 ml. (1.0 mole).

Attempted Dehydrogenation of Methyl Khusenate and Methyl Isokhusenate—Each of the esters was mixed with twice its weight of selenium and refluxed at 300 ± 10° in an atmosphere of nitrogen. The resultant products were extracted with hexane and chromatographed as described below.

Products from Methyl Khusenate—The reaction mixture (reaction time: 4 hr.) from methyl khusenate (100 mg.) was chromatographed on grade I alumina (5.5 Gm.). The following fractions were obtained: hexane (8 ml.), 13.1 mg.; hexane (24 ml.), 3.8 mg.; hexane (96 ml.), 1.9 mg.; benzene (32 ml.), 13.4 mg.

Products from Methyl Isokhusenate—Reaction of this ester (70 mg.) was allowed to proceed for 9 hr. The products were chromatographed on grade I alumina (5 Gm.) and three fractions were collected: hexane (8 ml.), 2.7 mg.; hexane (64 mg.), 0.3 mg.; hexane–benzene (4:1, 72 ml.), 4.4 mg.

Attempted Dehydrogenation of Crude Acid Mixture—*Employing Selenium*—The crude acid (356 mg.) was allowed to react with selenium for 14 hr. under experimental conditions described above for esters. The acidic constituents present in the reac-

tion mixture were separated as methyl esters employing Amberlite IRA-400. Yields of the neutral and acidic fractions (isolated as methyl esters) were 197 mg. and 22.1 mg., respectively. The neutral fraction was purified by chromatography on grade I alumina (5 Gm.) when a colorless hydrocarbon fraction (72 mg.) was obtained.

Employing Metal Catalysts—The experiment was repeated with the crude acid (250 mg.), employing 5% Pt on alumina (50 mg.) as the dehydrogenating agent. Analogous processing of the reaction mixture yielded a neutral oil (148 mg.) and methyl esters (41 mg.). Chromatography of the neutral fraction on alumina gave hydrocarbons (58 mg.). I.R. and U.V. analyses showed the hydrocarbon fraction did not undergo any chemical change on further refluxing with 5% Pd-on-carbon (31 mg.) for 2 hr. at 320–350°.

Characterization of the Hydrocarbon Fractions Obtained by Dehydrogenation Experiments—Similar hydrocarbon fractions (I.R. spectroscopy and gas chromatography) were obtained in each of the dehydrogenation experiments. The main constituent (79%) of the hydrocarbon fraction obtained by Se-dehydrogenation of the acids was isolated by gas chromatography on a Reoplex 400 column. It possessed the following characteristics: relative retention times on Reoplex 400 (column temperature, 200°) and SE-30 (column temperature, 185°): 0.49 and 1.76, respectively (reference standard: naphthalene; retention times: 6.70 min. and 1.65 min., respectively). NMR peaks at 1.47, 1.02, and 0.98 δ. Mol. wt. (mass spectrum) 190.

Ozonolysis of Methyl Khusenate—Methyl khusenate (1.00 Gm.) was dissolved in ethyl acetate (5 ml.), cooled to 0°, and ozonized using a Gallenkamp ozone apparatus GE-150. The ozonide was decomposed with hydrogen in the presence of Pd-C and the product treated with Amberlite IRA-400 (5 Gm.) in accordance with the procedure described above. The neutral fraction (918 mg.) obtained was purified by chromatography on grade III alumina (50 Gm.) using hexane–benzene mixture (1:1) as solvent and recrystallized from ethanol. Yield: 52% (by GLC), m.p. 102–103°.

Anal.—Calcd. for $C_{15}H_{22}O_3$: C, 71.95; H, 8.86. Found: C, 71.97; H, 8.87.

Infrared absorption bands (CCl_4 solution) at 1728, 1708, 1458, 1430, 1388, 1356, 1300, 1195, 1164, and 1042 cm.$^{-1}$.

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Drug Standards

New Electromechanical Method for the Assay of Heparin *In Vitro*

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A rapid, simple, and accurate *in vitro* assay for heparin employing a diatomaceous silicate modified plasma and a precision coagulation timer is reported. The method reveals standard deviations of ±2.5 sec. from the average clotting times with a coefficient of variation of about 2 per cent. Sodium heparin U.S.P., heparin reference standard lot H, and the second international standard for heparin have been successfully assayed by this method. The assays have also revealed the stability of sodium heparin to autoclaving and high energy electron irradiation.

WALTON *et al.* (1) have recently reviewed current methods of heparin assay. One of the most commonly employed methods is that of Reinert and Winterstein (2) which is the basis for the U.S.P. heparin assay procedure. A second method is that of Adams (3) which is prescribed by the British Pharmacopoeia. There exist numerous other methods which are essentially modi-

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