ard solution of DL-leucine turbidimetrically on a Coleman Model 11 Spectrophotometer at 600 mμ. The organism employed was *Lactobacillus arabinosus 17-5*, which responds only to the L-form of leucine. The medium employed was that of Kuiken, *et al.*[25]

Within the probable limits of error of the assay, the identity of the four leucylleucines was established. The L-L assayed 90–100% L-leucine, the L-D and D-L each 50% L-leucine (±5%), and the D-D hydrolyzate gave 0- < 1% L-leucine.

Partially Racemized Casein Fractions.—Smaco vitamin-free casein was partially racemized by the procedure of Dakin.[12] The $[\alpha]^{27}D$ for the original casein, the acid-precipitable racemized casein and the water-soluble caseose (precipitated by ammonium sulfate) were, respectively: $-104.7° \pm 0.8°$, $-52.0° \pm 0.8°$, and $-37.5° \pm 2.0°$ (0.3 g. in 25 ml. 0.5 *N* sodium hydroxide solution).

Inhibition Experiments.—The leucylleucines and leucines were first tested against *E. coli* in a dilution series. Twenty mg. of each compound was weighed into a small test-tube. Two ml. of nutrient broth[26] was added to each tube and solution was effected by warming. Half of this was mixed with medium in another tube, and this process repeated through a series of a total of six tubes. The tubes were plugged with cotton and autoclaved for ten minutes at 15 lb. steam pressure. The tubes were then inoculated from a fresh subculture of *E. coli*.

After twenty-five hours of incubation at 37°, the turbidities of the cultures were assessed in a Coleman Model 11 spectrophotometer at 650 mμ. The results are presented in Table I. The experiment was repeated with a synthetic medium consisting of 0.5% disodium phosphate, 0.5% dipotassium phosphate, 0.5% ammonium chloride, 0.02% magnesium sulfate and 0.5% glucose, brought to *p*H 7 with phosphoric acid. The visual results were similar to those in nutrient broth; only D-leucine at 10 mg./ml. showed total inhibition. No inhibition was found for any of the peptides tested against *L. arabinosus 17-5* in a yeast extract medium[27] with all other conditions the same as in the *E. coli* experiments. Because of the conceivability of racemization of the D-peptides during autoclaving,[28] the experiment of Table I was repeated with the glucose added aseptically after autoclaving, and again with the glucose replaced by glycerol. In both cases the results were identical with those in synthetic medium.

At concentrations of 10 mg./ml. of added racemized casein and of caseose, *E. coli* grew under the experimental conditions given above. *E. coli* was grown for the other inhibition experiments with nutrient broth as above, except for the added casein or racemized preparations. The racemized casein was dissolved in 2 *N* sodium hydroxide solution, neutralized, and Seitz-filtered for the tests. There was no inhibition of *L. arabinosus 17-5* by racemized casein at a concentration of 3.5 mg./ml. nor by caseose at a concentration of 15 mg./ml. in yeast extract medium at 37° for seventy-two hours.

A corresponding set of experiments with racemized gelatin (not isolated) gave similar results.

Experiments on Support of Growth.—For experiments on support of growth, casein, racemized casein, and caseose were made up in concentrations of 100 mg./ml. of 0.8% sodium carbonate solution containing traces of calcium chloride, magnesium sulfate and trisodium phosphate. No other protein was present. *E. coli* was the organism used. The results are presented in Table II.

Tests of replaceability of L-leucine by the leucylleucines were run in leucine-free synthetic medium[25] inoculated with *L. arabinosus*. The results are presented in Table III.

(25) Kuiken, Norman, Lyman, Hale and Blotter, *J. Biol. Chem.*, **151**, 615 (1943).

(26) Difco Laboratories, "Difco Manual," Detroit, Mich., 1943, p. 42.

(27) Difco Laboratories, "Difco Manual," Detroit, Mich., 1943, p. 178.

(28) Fling and Fox, *J. Biol. Chem.*, **160**, 329 (1945).

Summary

Racemized casein and caseose, containing numerous D-amino acid residues per molecule, failed to inhibit the growth of cultures of *Escherichia coli* or *Lactobacillus arabinosus 17-5*. None of the four isomeric leucylleucines showed as much antibacterial activity as D-leucine. The relationship of these experiments to the antibacterial activity of D-amino acids and of the antibiotics which are D-amino acid derivatives has been discussed.

The replaceability of L-leucine by leucylleucine isomers, in the medium for *L. arabinosus 17-5*, has been studied. The L-residues of L-leucyl-L-leucine and D-leucyl-L-leucine were fully utilized. None of the residues of L-leucyl-D-leucine nor of D-leucyl-D-leucine were available to this organism.

AMES, IOWA RECEIVED JANUARY 31, 1948

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

Action of Heat on D-Fructose. Isolation of Diheterolevulosan and a New Di-D-fructose Dianhydride

BY M. L. WOLFROM AND MARY GRACE BLAIR[1]

It is probable that the well-established tendency of D-fructose to form bimolecular cyclic anhydrides[2] accounts for some of the molasses formation occurring in cane sugar house processing. Such a view has been expressed by Sattler and Zerban.[3] These authors have investigated the non-fermented products formed on heating a concentrated aqueous solution of D-fructose. They established that the complex mixture obtained closely approximated in composition that required for a mixture of isomeric di-D-fructose dianhydrides. This work has now been repeated in our laboratory and the non-fermented products have been subjected to separation by chromatography on clay, a procedure established by Lew, Wolfrom and Goepp.[4] Two crystalline products were isolated in pure form and characterized. The one was the diheterolevulosan of Pictet and Chavan[5]

(1) Sugar Research Foundation Fellow of The Ohio State University Research Foundation (Project 190).

(2) For a review of the di-D-fructose dianhydrides see Emma J. McDonald, *Advances in Carbohydrate Chem.*, **2**, 253 (1946).

(3) L. Sattler and F. W. Zerban, *Ind. Eng. Chem.*, **37**, 1133 (1945).

(4) B. W. Lew, M. L. Wolfrom and R. M. Goepp, Jr., THIS JOURNAL, **67**, 1865 (1945); **68**, 1449 (1946).

(5) A. Pictet and J. Chavan, *Helv. Chim. Acta*, **9**, 809 (1926).

and the other, designated diheterolevulosan II, was an isomeric substance (m. p. 250–252°, dec.; $[\alpha]^{25}D$ −39° in water) not identical with any known di-D-fructose dianhydride.

Pictet and Chavan[5] prepared their crystalline diheterolevulosan by treating D-fructose at low temperatures with concentrated hydrochloric acid. This work was repeated in our laboratory in order to obtain material for comparative purposes. The complex reaction product was subjected to chromatographic separation and the diheterolevulosan of Pictet and Chavan[5] was obtained with constants in agreement with those cited by these workers. In addition, diheterolevulosan II was obtained from this source. The crystalline hexaacetate of diheterolevulosan was prepared and was found to have properties in agreement with those described by Schlubach and Behre.[6] The hexaacetate of diheterolevulosan II was likewise obtained in crystalline form.

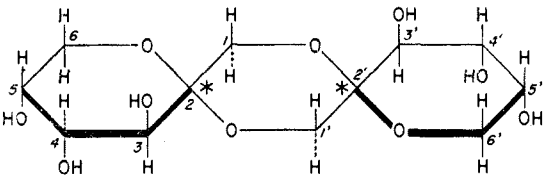
Pictet and Chavan[5] isolated an amorphous fraction which they termed "heterolevulosan" and which they considered to be a monomolecular anhydride of D-fructose. A material of similar properties was obtained on following their procedure. It was easily demonstrated by chromatographic methods, however, that this fraction was a complex mixture whose main component was the diheterolevulosan II. There thus exists no evidence for a monomolecular anhydride of D-fructose or for its "dimerization"[3,5] to a di-D-fructose dianhydride. Like "glutose,"[3] "heterolevulosan" has no existence in fact and the name should be stricken from the chemical literature.

Schlubach and Behre[6] methylated diheterolevulosan to a crystalline hexamethyl ether from which was obtained on acid hydrolysis a sirupy trimethyl-D-fructose, $[\alpha]^{20}D$ −73.5° in water, which formed an oily phenylosazone without demethylation. McDonald and Jackson[7] noted that diheterolevulosan absorbed four moles of periodate. To this we add that two moles of formic acid and no formaldehyde are produced in this oxidation. Upon these data the structure I (Fig. 1), di-D-fructopyranose 1,2′:2,1′-dianhydride, is evinced for diheterolevulosan. The methylation data do not meet the exacting criteria of crystallinity and are therefore inadequate. Fortunately, the periodate work is definitive and leads uniquely to formula I. The structure of diheterolevulosan is thus established save for the assignment of configurations to the anomeric spirane carbon atoms.

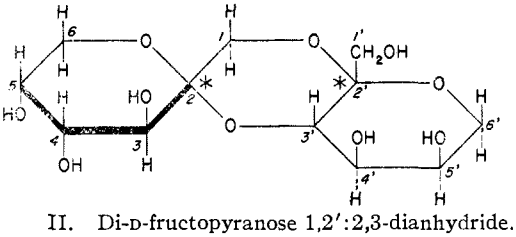
We can presently adduce for the structure of diheterolevulosan II only the results of periodate analysis which are that three moles of oxidant are consumed with the concomitant formation of one mole of formic acid and no formaldehyde. Formulas II and III satisfy these demands and for the sake of simplicity we favor III. Further data are required for an unequivocal proof of structure.

(6) H. H. Schlubach and C. Behre, *Ann.*, **508**, 16 (1934).

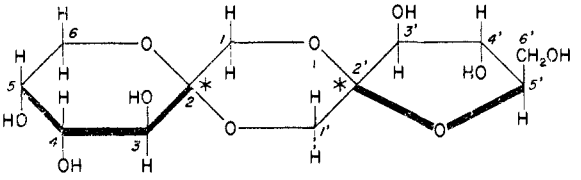
(7) Emma J. McDonald and R. F. Jackson, *J. Research Natl. Bur. Standards*, **35**, 497 (1945).



I. Diheterolevulosan (di-D-fructopyranose 1,2′:2,1′-dianhydride).



II. Di-D-fructopyranose 1,2′:2,3-dianhydride.



III. D-Fructopyranose-D-fructofuranose 1,2′:2,1′-dianhydride.

*Anomeric configuration unknown.

Fig. 1.—Di-D-fructose dianhydrides.

Experimental

Treatment of D-Fructose with Hydrochloric Acid and Recovery of the Crude Reaction Products.—Following Pictet and Chavan,[5] D-fructose (100 g.) was dissolved at 0° in 400 g. of concentrated hydrochloric acid (sp. gr. 1.19 at 15.56°) and the solution was maintained at 0° for seventy-two hours. Neutralization was then effected with basic lead carbonate and the dissolved lead chloride was removed from the filtered solution by ion exchange on Amberlites IR-100 and IR-4.[8] Unchanged D-fructose was removed from the solution (1500 ml.) by fermentation with 20 g. of baker's yeast (Fleischmann) at 30° for three days. Upon the cessation of fermentation, the yeast was removed by centrifugation and filtration through a filter-aid. The filtrate was deionized with Amberlites IR-100 and IR-4[8] and concentrated to a sirup which was dried by the addition of absolute ethanol and its subsequent removal by distillation under reduced pressure. Crystallization was effected from methanol; yield 28 g. in two crops (fraction A). The filtrate (150 ml.) was added dropwise to 1.5 liters of anhydrous ether and the precipitate so formed was removed by filtration; yield 4.7 g. (fraction B), $[\alpha]^{24}D$ −61.0° (*c* 4, water). The material was an amorphous, somewhat hygroscopic, white powder.

Diheterolevulosan from Fraction A.—Fraction A (28 g.) above was recrystallized several times from water to yield pure diheterolevulosan; yield 5.9 g., m. p.[9] 261–263° (dec.), $[\alpha]^{18}D$ −45.8° (*c* 4, water). This material was chromatographically pure; it showed only one zone when chromatographed according to the extrusion procedure described below. Pictet and Chavan[5] cite for

(8) Products of the Resinous Products and Chemical Co., Philadelphia, Pennsylvania.

(9) Unless otherwise noted, all melting points are uncorrected and were taken on a modified Berl–Kullmann block as described by F. W. Bergstrom, *Ind. Eng. Chem., Anal. Ed.*, **9**, 340 (1937). The substances were heated as rapidly as possible from room temperature to near the melting point.

diheterolevulosan the constants: m. p. 266–267°; $[\alpha]^{18}D$ −43.5° (*c* 4, water).

Anal. The substance (1 mole) consumed 4.2 moles of sodium metaperiodate and formed 2.0 moles of formic acid and no formaldehyde.

Acetylation of this material (0.5 g.) with pyridine (7 ml.) and acetic anhydride (4.5 ml.) by shaking mechanically at room temperature for four to five days, yielded hexaacetyldiheterolevulosan (recrystallized from absolute ethanol); yield 0.8 g., m. p. 172.5–173.5° (cor.), $[\alpha]^{28}D$ −59.0° (*c* 1, U. S. P.[10] chloroform). Schlubach and Behre[6] cite for hexaacetyldiheterolevulosan the constants: m. p. 171–173°; $[\alpha]^{20}D$ −59.1° (*c* 1, chloroform).

Diheterolevulosan II from Fraction A.—The aqueous mother liquor from the above-described preparation of diheterolevulosan was concentrated to a sirup; yield 22 g. An amount of 11 g. of this sirup was dissolved in 8 ml. of water, made into a slurry with clay–Celite[11] (5:1 by wt.) and treated with 200 ml. of 95% ethanol. The mixture was added to the top of a 2-liter pharmaceutical percolator packed with 1 kg. of the same clay–Celite mixture and saturated with 95% ethanol. To this mixture was added 95% ethanol until 6.3 liters of effluent was obtained. Evaporation of the filtered effluent yielded a sirup (fraction A-1). Additional development with 95% ethanol removed a negligible amount of material (very weak Molisch test). Changing of the developer to 80% ethanol slowly removed further material. From 5 liters of effluent there was recovered, in the manner described above, crystalline material identified as diheterolevulosan; yield 1.0 g., $[\alpha]^{27}D$ −45.4° (*c* 4, water).

Fraction A-1 was dissolved in water and deionized with Amberlite IR-100 and IR-4.[8] The deionized solution was concentrated under reduced pressure to a thick sirup and crystallized by the addition of methanol; yield 7.2 g., $[\alpha]^{26}D$ −39.5° (*c* 4, water). The material was recrystallized by solution in water, concentration to a thick sirup and addition to this of absolute methanol. This product contained some inorganic material which was removed by solution of the organic crystals in boiling absolute methanol and filtration. The solvent was removed and the procedure was repeated with 90% ethanol. Crystallization was effected by concentration of the dilute ethanol solution; yield 6.4 g., m. p. 250–252° (dec.), m. p. 239–243° on admixture with diheterolevulosan of m. p. 261–263° (dec.), $[\alpha]^{28}D$ −39° (*c* 4, water). The substance reduced Fehling solution after acid hydrolysis with 5% sulfuric acid for thirty minutes at 70°. It was chromatographically pure, showing only one zone when chromatographed on clay according to the procedure detailed below.

Anal. Calcd. for $C_{12}H_{20}O_{10}$: C, 44.44; H, 6.22. Found: C, 44.28; H, 6.18. Periodate analysis: the substance (1 mole) consumed 3.0 moles of sodium metaperiodate and formed 1.0 mole of formic acid and no formaldehyde.

Hexaacetyldiheterolevulosan II.—This substance was prepared according to the procedure described above for hexaacetyldiheterolevulosan except that solution was readily effected in the acetylating mixture and two days standing at room temperature without shaking sufficed. It was obtained crystalline from absolute ethanol; m. p. 123–124°; $[\alpha]^{29}D$ −41.5° (*c* 4, U. S. P.[10] chloroform).

Anal. Calcd. for $C_{12}H_{14}O_{10}(CH_3CO)_6$: C, 49.99; H, 5.60; CH_3CO, 10.4 ml. 0.1 *N* NaOH per 100 mg.; mol. wt., 577. Found: C, 50.11; H, 5.72; CH_3CO, 10.4 ml.; mol. wt. (Rast), 604.

Deacetylation was effected by the procedure of Kunz and Hudson[12] (sodium hydroxide in dilute acetone at −15°). Acetone was removed from the neutralized solution by distillation under reduced pressure and inorganic ions were removed by passage through ion exchange columns (Amberlites IR-100 and IR-4[8]). Concentration to a thick sirup followed by methanol addition yielded the original crystalline diheterolevulosan II; m. p. 250–252°, $[\alpha]^{23}D$ −38.7° (*c* 4, water).

Investigation of Fraction B.—Fraction B above (4.7 g., $[\alpha]^{24}D$ −61° in water) corresponds to the amorphous material described by Pictet and Chavan[5] as "heterolevulosan," $[\alpha]^{24}D$ −66° (*c* 3, water), and considered by them to be a six carbon anhydro-D-fructose. Three grams of this fraction was dissolved in 45 ml. of water and 255 ml. of dioxane (distilled from sodium) was added. The resultant solution was chromatographed in 5-cc. portions on 25 g. of clay–Celite (5:1) in tapered tubes (23 mm. diam. at bottom and 25 mm. diam. at top). Development was effected with 70 ml. of 95% dioxane. Extrusion of the chromatograms and streaking with the alkaline permanganate indicator (1% potassium permanganate in 2 *N* sodium hydroxide) located three zones in each: C, 6–18 mm. from the top; D, 45–70 mm.; E, 95–125 mm. No significant amount of material was present in the effluents. The sectioned fractions were eluted with 80% ethanol. The collected material from the C zones yielded a small amount of crystalline substance that is under further investigation; m. p. 261–263° (dec.), $[\alpha]^{25}D$ −90° (*c* 4, water). Zone E yielded a sirup; yield 0.3 g. Zone D contained diheterolevulosan II; 0.8 g., $[\alpha]^{23}D$ −37° (*c* 4, water). Ash-free material was obtained on recrystallization as described above; m. p. 248–250° (dec.) undepressed on admixture with the material described above, $[\alpha]^{24}D$ −37° (*c* 4, water).

Anal. Calcd. for $C_{12}H_{20}O_{10}$: C, 44.44; H, 6.22. Found: C, 44.08; H, 6.08.

Subjection of an Aqueous Solution of D-Fructose to the Action of Heat and Recovery of the Reaction Products.—Following the heat treatment procedure of Sattler and Zerban,[3] 100 g. of D-fructose was dissolved in 25 ml. of water and the resultant solution was refluxed gently for sixteen hours. The solution was then diluted to 700 ml. with sterile water and fermented and deionized as described previously. The resultant dried sirup was dissolved in 200 ml. of dry methanol and the solution added dropwise to 2 liters of dry acetone. The resultant flaky, cream colored, somewhat hygroscopic, amorphous solid was removed by filtration; yield 26 g., $[\alpha]^{24}D$ −23° (*c* 4, water). The precipitate was redissolved in water, treated with decolorizing charcoal, and the solution again evaporated to dryness under reduced pressure. The dry residue was further purified by two precipitations effected by the dropwise addition of its methanolic solution to acetone; yield 18 g., $[\alpha]^{24}D$ −32.6° (*c* 4, water). An amount of 16.4 g. of this material was crystallized from anhydrous methanol; yield 1.1 g. of crystals (fraction F), $[\alpha]^{22}D$ −37° (*c* 4, water). The mother liquor material will be designated fraction G.

Fraction F (1.1 g.) was dissolved in 15 ml. of water to which was then added 85 ml. of purified dioxane. This solution was chromatographed in 5-cc. portions exactly as described above for fraction B. Two zones were obtained: H, 8–23 mm. from the top; I, 36–72 mm. The collected material from the H zone yielded crystals on concentration of the eluate; yield 0.49 g., $[\alpha]^{30}D$ −48° (*c* 4, water). Pure material was obtained on several recrystallizations effected by solution in a minimum amount of hot water and addition of ethanol. It was identified as diheterolevulosan; m. p. 261–263° (dec.) undepressed on admixture with the above-described material from the acid treatment of D-fructose, $[\alpha]^{24}D$ −45.0° (*c* 4, water).

Anal. Calcd. for $C_{12}H_{20}O_{10}$: C, 44.44; H, 6.22. Found: C, 44.23; H, 5.96.

The hexaacetate was prepared as described above; m. p. 172.5–173.5° (cor.) undepressed on admixture with material from the acid treatment of D-fructose, $[\alpha]^{28}D$ −58.0° (*c* 1, U. S. P.[10] chloroform).

(10) United States Pharmacopoeia.

(11) The clay employed was Florex XXX, a fuller's earth type of clay, produced by the Floridin Co., Warren, Pennsylvania. The Celite (no. 535) was a siliceous filter-aid produced by Johns-Manville Co., New York, N. Y. The mixture was purified by solvent extraction as described by Lew, Wolfrom and Goepp.[4]

(12) A. Kunz and C. S. Hudson, THIS JOURNAL, **48**, 1982 (1926).

The collected material from the above-described zone I was a sirup that crystallized on the addition of anhydrous methanol; yield 0.19 g. (fraction J), $[\alpha]^{29}D$ −37.6° (*c* 4, water). This material was combined below to form fraction K.

An amount of 12 g. of fraction G above was dissolved in 180 ml. of water and to this was added 1020 ml. of purified dioxane. The resultant solution was chromatographed in 5-ml. portions as described above for fraction B. Zones were obtained that were located in the same positions as those found in fraction B. The collected material from the bottom zones yielded a sirup that was not further investigated; yield 1.4 g. The material from the top zones was found to contain diheterolevulosan, identified by optical rotation, which was isolated as described above; yield 0.32 g. The collected material from the middles zones crystallized in part from methanol; yield 1.2 g., $[\alpha]^{28}D$ −39.0° (*c* 4, water). This crystalline material was combined with fraction J above to form fraction K which was further purified from methanol and 90% ethanol and was identified as diheterolevulosan II; m. p. 250–252° (dec.) undepressed on admixture with material obtained by the acid treatment of D-fructose, $[\alpha]^{28}D$ −39.0° (*c* 4, water).

Anal. Calcd. for $C_{12}H_{20}O_{10}$: C, 44.44; H, 6.22. Found: C, 44.31; H, 6.20.

The material was non-reducing toward Fehling solution and yielded the above-described hexaacetate of diheterolevulosan II.

Summary

1. Diheterolevulosan (di-D-fructopyranose 1,-2′:2,1′-dianhydride, I) and a new di-D-fructose dianhydride, designated diheterolevulosan II, have been isolated in crystalline form by chromatographic methods from the products obtained by the action of heat or of hydrogen chloride upon concentrated aqueous solutions of D-fructose.

2. Periodate analysis of diheterolevulosan II (II) favors, but does not prove, a 1,2′:2,1′-dianhydride structure (III) formed between a mole of D-fructopyranose and one of D-fructofuranose.

3. The amorphous "heterolevulosan" of Pictet and Chavan is shown by chromatographic methods to be a complex mixture, the principal constituent of which is diheterolevulosan II.

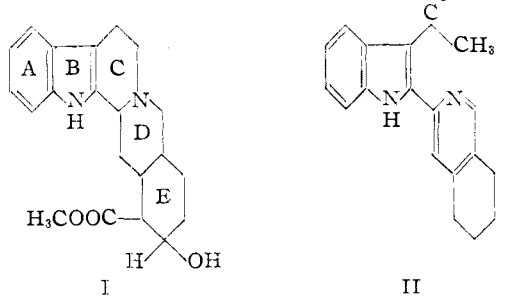
COLUMBUS, OHIO RECEIVED FEBRUARY 16, 1948

[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

The Structure of Ketoyobyrine

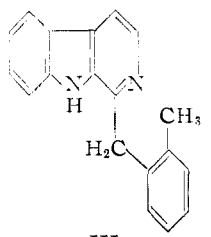
BY R. B. WOODWARD AND BERNHARD WITKOP

The selenium dehydrogenation of yohimbine (I) gives two bases, tetrahydroisoyobyrine (II), yobyrine (III) and ketoyobyrine, a neutral substance of the formula $C_{20}H_{16}ON_2$. The study of the



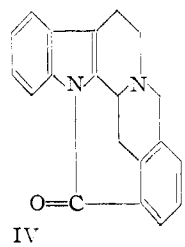
I

II

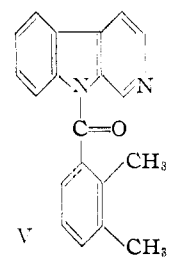


III

basic products was of primary importance in deducing the structure of yohimbine, and the structures of the bases have been established beyond question.[1,2] On the other hand, no satisfactory formulation of ketoyobyrine has been forthcoming. Scholz[3] originally put forward the expression (IV); the facts that ketoyobyrine is optically inactive, that it is the product of a drastic dehydrogenation reaction, and in particular that it has no basic properties, are incompatible with that formula. The outstanding phenomenon in the chem-



IV



V

istry of ketoyobyrine is the smooth cleavage of the molecule by amyl alcoholic potassium hydroxide to hemellitylic acid and norharmane.[3,4] This behavior has been adduced in support of an alternative formula (V),[2] which, however, still cannot be reconciled with the neutral character of the molecule.

In this communication, it is shown that in fact ketoyobyrine[5] has the structure (VI). This formula was deduced from that of yohimbine (I) on the basis of these considerations: (i) when yohimbic acid is heated with selenium, loss of the

(1) Scholz, *Helv. Chim. Acta*, **18**, 923 (1935).

(2) Witkop, *Ann.*, **554**, 83 (1943); *cf.* Clemo and Swan, *J. Chem. Soc.*, 617 (1946); Julian, *et al.*, THIS JOURNAL, **70**, 180 (1948).

(3) Scholz, Diss. Eidgen. Techn. Hochschule, Zürich, 1934.

(4) Mendlik and Wibaut, *Rec. trav. chim.*, **50**, 91 (1931).

(5) It is clear that the term ketoyobyrine is a misnomer, but in view of long-established usage, we feel that a change is not desirable.