[CONTRIBUTION FROM THE LABORATORY OF CHEMISTRY AND CHEMOTHERAPY, EXPERIMENTAL BIOLOGY AND MEDICINE INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

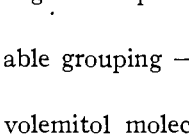
The Oxidation of Volemitol by Acetobacter suboxydans and by Acetobacter xylinum

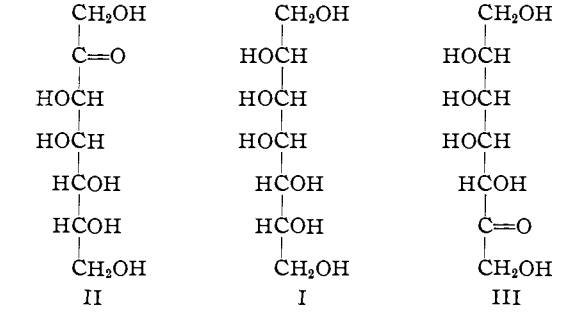
BY LAURA C. STEWART, NELSON K. RICHTMYER AND C. S. HUDSON

Volemitol (I) was discovered by Bourquelot[1] in the mushroom *Lactarius volemus* Fr., by Bougault and Allard[2] in the roots of several species of *Primula*, and more recently by Asahina and Kagitani[3] in the lichen *Dermatocarpon miniatum* (L.) Mann. From the synthetic side this same heptitol has been described by Peirce[4] who called it D-β-mannoheptitol because he had obtained it from D-β-mannoheptose by reduction with sodium amalgam; by LaForge,[5] who isolated it as one of the reduction products of naturally occurring D-mannoheptulose; and by LaForge and Hudson,[6] who called it α-sedoheptitol when they found it to be one of the reduction products of naturally occurring sedoheptulose. LaForge[7] showed that α-sedoheptitol was identical with volemitol, and Ettel[8] later proved the identity of volemitol with D-β-mannoheptitol of known configuration.[4] By our present system of nomenclature, volemitol (I) may be named either D-manno-D-*talo*-heptitol or D-altro-D-*manno*-heptitol.

In a recent contribution from this Laboratory[9] it was established that the "phenyl-volemosazone" which Fischer[10] obtained from the products of oxidation of volemitol by sodium hypobromite was D-mannoheptose phenylosazone, and it was suggested accordingly that the name "volemose" might well be discarded or used only in a historical way. In accord with a previously announced intention,[9] we have now repeated Bertrand's[11] biochemical oxidation of volemitol with *Acetobacter xylinum* and have identified "volemulose," the sirupy ketone sugar formed. Very recently, Ettel and Liebster[12] showed that *A. suboxydans* oxidized volemitol to a mixture of D-mannoheptulose and D-altroheptulose (= sedoheptulose) and estimated that the two sugars were produced in equal amounts. The present independently conceived experiments agree with these results, but they disprove Ettel and Liebster's inference that *A. xylinum* and *A. suboxydans* give the same products from volemitol.

(1) E. Bourquelot, *Bull. soc. mycologique France*, **5**, 132 (1889); *J. pharm. chim.*, [6] **2**, 385 (1895).
(2) J. Bougault and G. Allard, *Compt. rend.*, **135**, 796 (1902); *Bull. soc. chim.*, [3] **29**, 129 (1903).
(3) Y. Asahina and M. Kagitani, *Ber.*, **67**, 804 (1934).
(4) G. Peirce, *J. Biol. Chem.*, **23**, 327 (1915).
(5) F. B. LaForge, *ibid.*, **28**, 511 (1917).
(6) F. B. LaForge and C. S. Hudson, *ibid.*, **30**, 61 (1917).
(7) F. B. LaForge, *ibid.*, **42**, 375 (1920).
(8) V. Ettel, *Collection Czechoslov. Chem. Commun.*, **4**, 504 (1932).
(9) W. T. Haskins and C. S. Hudson, THIS JOURNAL, **69**, 1370 (1947).
(10) E. Fischer, *Ber.*, **28**, 1973 (1895).
(11) (a) G. Bertrand, *Compt. rend.*, **126**, 763 (1898); (b) *Bull. soc. chim.*, [3] **19**, 347 (1898); (c) *Ann. chim.*, [8] **3**, 209, 287 (1904).
(12) V. Ettel and J. Liebster, *Collection Czechoslov. Chem. Commun.*, **14**, 80 (1949).

Because *Acetobacter suboxydans*, in contrast to *A. xylinum*, had previously been found to give excellent yields of ketone sugars from most of the polyhydric alcohols which it attacks, we decided to study first the behavior of *A. suboxydans* toward volemitol. According to the specificity rule of Bertrand[13] for *A. xylinum*, as extended by Hann, Tilden and Hudson[14] to *A. suboxydans*, we should expect that the latter organism would oxidize volemitol (I) to D-mannoheptulose (II), D-altroheptulose (=sedoheptulose) (III), or to a mixture of these two ketoses. Experiments showed that volemitol was indeed oxidized readily by *A. suboxydans*, and reducing sugar was produced in nearly theoretical yield. The resulting solution, from 9 g. of volemitol, was deproteinized, deionized, and concentrated to a sirup from which we were able to isolate 3.8 g. of crystalline D-mannoheptulose. The mother liquor was heated with dilute sulfuric acid to convert any sedoheptulose to its anhydride, and we could then obtain 2.2 g. of crystalline sedoheptulosan. Thus, as might be expected from the presence of the favorable grouping  at each end of the volemitol molecule, both D-mannoheptulose and D-altroheptulose were formed by the action of *A. suboxydans*.



II D-Mannoheptulose

I Volemitol; α-Sedoheptitol; D-β-Mannoheptitol; D-Manno-D-*talo*-heptitol; D-Altro-D-*manno*-heptitol

III Sedoheptulose (D-Altroheptulose)

On the other hand, the oxidation of 4 g. of volemitol by *A. xylinum* proceeded very slowly. A heavy pellicle of cellulosic material was formed, and titration of the solution showed that only 29% of the expected reducing sugar was formed even after fifty-five days. The solution, freed from

(13) G. Bertrand, ref. 11a, 11b and 11c, p. 202.
(14) R. M. Hann, E. B. Tilden and C. S. Hudson, THIS JOURNAL, **60**, 1201 (1938).

protein, ionizable material, and 2.2 g. of unchanged volemitol, was concentrated to a sirup which showed no tendency to crystallize when inoculated with D-mannoheptulose. It was heated with dilute sulfuric acid, with a resulting change in rotation from positive to negative, and 0.22 g. of crystalline sedoheptulosan was isolated. The mother liquor was acetylated and chromatographed, but no evidence could be found to indicate the presence of D-mannoheptulose.[14a] The conclusion is that *A. xylinum* appears to attack only one end of the volemitol molecule to produce a ketoheptose, and that Bertrand's sirupy "volemulose" was in reality the first description of the sugar we now know as D-altroheptulose (=sedoheptulose). The phenylosazone of "volemulose," reported by Bertrand[11a,b] to melt at 205–207° is to be identified as D-altroheptose phenylosazone, even though the melting point of the latter is known to be somewhat lower, namely, 197°[6] or 194–195°.[15]

Experimental

Volemitol.—The material used in these experiments was prepared by Dr. Raymond M. Hann and Mr. John T. Sipes, of this Laboratory, by the reduction of sedoheptulose sirups with sodium amalgam.[16] The recrystallized product melted at 152°.

Oxidation of Volemitol by Acetobacter suboxydans.—After some preliminary experiments had been completed, the following procedure was adopted for the oxidation of the 10-g. sample. A medium was prepared to contain 0.5% of Difco yeast extract, 0.3% of potassium dihydrogen phosphate, 0.05% of D-glucose and 2% of volemitol after dilution to 500 ml. with distilled water. This solution was distributed among five 500-ml. Erlenmeyer flasks, sterilized, and each flask was inoculated with 0.3 ml. of a three-day-old culture of *A. suboxydans*[17] grown on a yeast extract and D-glucose medium. The mixture was incubated in an oven at 30°. The progress of the reaction was followed by deproteinizing 1-cc. aliquots with zinc sulfate and barium hydroxide according to Somogyi,[18] and determining reducing sugar in the filtrate by the method of Hagedorn and Jensen[19] as modified by Hanes.[20] After one hundred and sixty-two hours a reducing value equivalent to 18.1 mg. of mannoheptulose per ml. had been reached, and was unchanged after another twenty-four hours.

The remainder of the material in the five flasks was combined and deproteinized by the addition of 250 ml. of 5% aqueous zinc sulfate solution followed by an equivalent amount of aqueous barium hydroxide solution, so that the resulting reaction mixture was neutral to phenolphthalein. The clear, colorless filtrate was deionized by passage through columns of Amberlite IR-100 and IR-4B, and then concentrated *in vacuo* to 280 ml. At this point, 28 ml. of the solution, corresponding to about 1 g. of reducing sugars, was heated with 2 ml. of phenylhydrazine and 1 ml. of glacial acetic acid for two hours on the steam-bath. The resulting phenylosazone was filtered from the cooled solution, and washed successively with 10% acetic acid, water, ethanol and ether. The yellow, finely crystalline powder weighed 1.1 g. and melted at 180–187° with decomposition. Upon recrystallization from 75 ml. of ethanol the clusters of tiny, yellow needles (0.4 g.) melted at 177–185° with decomposition. The material is evidently a mixture of the phenylosazones[9] of D-altroheptose (194–195°) and D-mannoheptose (199–200°), and no further attempts were made to separate the components.

The main portion of the solution was concentrated *in vacuo* to a dry sirup which was taken up in methanol and allowed to concentrate in a desiccator. Upon inoculation at the thin-sirup stage, D-mannoheptulose separated in a yield of 2.8 g., with an additional 1.0 g. being obtained later. The product, after one recrystallization as prisms from methanol, weighed 2.2 g., melted at 151–152° and showed no depression of melting point when mixed with an authentic sample. The rotation, $[\alpha]^{20}D$ +29.1° in water (*c*, 2.5), was in agreement with the values +29.0 and +29.4° reported by LaForge.[5]

The filtrate from the D-mannoheptulose crystals was concentrated to a sirup which was dissolved in 30 ml. of *N* sulfuric acid and heated on the steam-bath for three hours. The solution was neutralized with excess barium carbonate, filtered, and concentrated *in vacuo* to a thick sirup. By dissolving the sirup in methanol and again concentrating, we obtained a total of 2.2 g. of the characteristic prisms of sedoheptulosan.[21] After one recrystallization from hot methanol the product had a rotation, $[\alpha]^{20}D$ −143° in water (*c*, 2), and melting point, 154–155°, in good agreement with the values −146° and 155–156° reported by LaForge and Hudson.[6] A mixture of our product with sedoheptulosan from *Sedum spectabile* showed no depression of melting point.

Oxidation of Volemitol by Acetobacter xylinum.—In view of the experience of Tarr and Hibbert,[22] preliminary studies were made to determine optimal cultural conditions for the growth of *A. xylinum* upon mannitol and perseitol as substrates. A small concentration of ethanol appeared to stimulate the oxidation to some extent, whereas D-glucose and sodium lactate had little or no stimulatory effect. The medium finally selected contained 0.5% of Difco yeast extract, 0.3% of potassium dihydrogen phosphate and 2% of volemitol after dilution to 200 ml. with distilled water. The solution was divided between two 500-ml. Erlenmeyer flasks, sterilized, and to each flask was added 0.7 ml. of ethanol; this was followed by an inoculum of five drops of a twenty-four-hour culture of *Acetobacter xylinum*[23] which had been grown on a yeast extract and D-glucose broth containing 10% of canned cherry juice. After thirty days' incubation at 30° the mixture contained the equivalent of 5.2 mg. of mannoheptulose per ml.; after forty days the value had risen to 5.8 mg./ml., and was unchanged after another fifteen days. The solution was separated from the pellicle of cellulosic material, deproteinized, deionized, and concentrated *in vacuo* to dryness. The solid residue was extracted several times with warm methanol, leaving 2.2 g. of unchanged volemitol. The concentrated methanol solution would not crystallize when inoculated with D-mannoheptulose. Accordingly, the sirup was heated on the steam-bath for three hours with 50 ml. of *N* sulfuric acid; the levorotatory solution was neutralized with excess barium carbonate, filtered, and concentrated to a dry sirup. A methanol extract then yielded 0.22 g. of crystalline sedoheptulosan which was identified, after one recrystallization from methanol, by its rotation of $[\alpha]^{20}D$ −146° in water

(14a) Note added Sept. 29, 1949.—In order to eliminate the possibility that D-mannoheptulose was formed and then oxidized further by *A. xylinum*, it has now been shown in a separate experiment that a 0.5% solution of this ketose was unaffected even by seven weeks' incubation with *A. xylinum* in the usual culture medium.

(15) The melting point reported by Bertrand may have been observed on the Maquenne block.

(16) A. T. Merrill, W. T. Haskins, R. M. Hann and C. S. Hudson, THIS JOURNAL, **69**, 70 (1947).

(17) American Type Culture Collection No. 621.

(18) M. Somogyi, *J. Biol. Chem.*, **160**, 69 (1945).

(19) H. C. Hagedorn and B. N. Jensen, *Biochem. Z.*, **135**, 46 (1923).

(20) C. S. Hanes, *Biochem. J.*, **23**, 99 (1929).

(21) Sedoheptulosan monohydrate has recently been obtained in this Laboratory by crystallization of sedoheptulosan from water or aqueous ethanol. In contrast to the anhydrous form it is stable in moist air. Further details will appear later.

(22) H. L. A. Tarr and H. Hibbert, *Can. J. Research*, **4**, 372 (1931).

(23) Obtained through the courtesy of Dr. Reese H. Vaughn of the University of California.

(*c*, 1.2), and its melting point and mixed melting point of 154–155°.

The mother liquor from the sedoheptulosan still would not crystallize when concentrated and inoculated with D-mannoheptulose. The 0.38 g. of sirup was acetylated with acetic anhydride and pyridine, to yield 0.57 g. of sirupy acetate. Using the technique of flowing chromatography which we had already found could be applied to the acetylated mother liquor of the *A. suboxydans* experiment, we dissolved the 570 mg. of this acetate in ether, poured it on a column containing 10 g. of alumina, and eluted it exhaustively with absolute ether to remove 363 mg. of levorotatory material. Further elution with mixtures of ether and ethyl acetate produced 152 mg., in five fractions, all of which were dextrorotatory, but none could be induced to crystallize when inoculated with hexaacetyl-α-D-mannoheptulose as did certain of the corresponding fractions from the *A. suboxydans* experiment. In this case the dextrorotatory material presumably consists of acetylated sedoheptulose which has not yet been obtained in crystalline form. The combined five fractions were deacetylated, but the small amount of sirup still would not crystallize when inoculated with mannoheptulose.

Summary

The action of *Acetobacter suboxydans* upon volemitol proceeds readily and nearly quantitatively to produce both D-mannoheptulose and D-altroheptulose (=sedoheptulose). This result is in agreement with the specificity rule of Hann, Tilden and Hudson for the action of *A. suboxydans*.

The action of *Acetobacter xylinum* upon volemitol is slow and incomplete; only one end of the molecule appears to be oxidized, and the sirupy ketose, which was first obtained by Bertrand and named "volemulose," has been identified as D-altroheptulose (=sedoheptulose).

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[CONTRIBUTION FROM THE IPATIEFF HIGH PRESSURE AND CATALYTIC LABORATORY, DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY]

Hydrogen Transfer. III.[1] Reaction of *p*-Ethyltoluene and *p*-Propyltoluene with Methylcyclohexene. Synthesis of Diarylalkanes

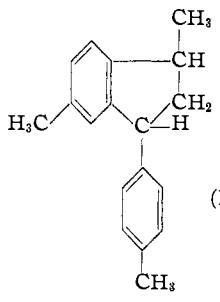
BY HERMAN PINES, D. R. STREHLAU[2] AND V. N. IPATIEFF

In a previous paper it has been shown that an abnormal reaction occurs when *p*-cymene is contacted with methylcyclohexene in the presence of either sulfuric acid or hydrogen fluoride.[3] Instead of the expected cycloalkylation of *p*-cymene, a hydrogen transfer was the main reaction; the methylcyclohexene acted as a hydrogen acceptor, forming methylcyclohexane, while *p*-cymene acted as a hydrogen donor yielding as the main product 1,3,3,6-tetramethyl-1-*p*-tolylindan.

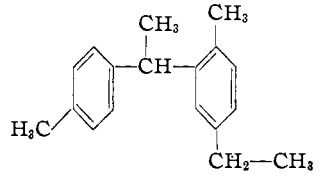
It was of interest to determine whether a para disubstituted benzene ring having an alkyl group containing more than one hydrogen atom on the carbon attached to the benzene ring would also yield products resulting from a hydrogen transfer reaction. For that reason *p*-xylene, *p*-ethyl- and *p*-propyltoluene reacted with methylcyclohexene in the presence of hydrogen fluoride and/or sulfuric acid.

p-Xylene, on reacting with methylcyclohexene in the presence of hydrogen fluoride, yielded only methylcyclohexyl-*p*-xylene. The formation of methylcyclohexane which serves as an indicator of a hydrogen transfer reaction was not observed.

Hydrogen transfer was the main reaction when *p*-ethyltoluene and methylcyclohexene in the molal ratio of two to one reacted in the presence of hydrogen fluoride. Forty-five per cent. of the methylcyclohexene was converted to methylcyclohexane and 20% to a compound corresponding to dimethyldicyclohexyl. Of the converted *p*-ethyltoluene 65% underwent a hydrogen transfer reaction to form a compound (Y) containing 18 carbon atoms boiling at 157° (6 mm.), $n^{20}D$ 1.5540; and 23% underwent condensation with methylcyclohexene, yielding probably 2-(1-methylcyclohexyl)-4-ethyltoluene[3a]; and remainder of the reacted *p*-ethyltoluene corresponded to a condensation product of methylcyclohexene with compound (Y). It was thought at first that *p*-ethyltoluene reacted with methylcyclohexene in a manner similar to the reaction of *p*-cymene and methylcyclohexene, and that 3,6-dimethyl-1-*p*-tolylindan (I), would be formed.



(I)



(II)

It was found however that the physical constants, solid derivatives and infrared absorption spectra (Graph I) of synthetic (I) did not correspond to the compound (Y) (Graph II).

In line with the mechanism proposed for the hydrogen transfer reactions described previously,[1] it

(1) For paper II of this series see H. Pines, A. Weizmann and V. N. Ipatieff, THIS JOURNAL, **70**, 3859 (1948).

(2) Universal Oil Products Company Predoctorate Fellow (1945–1947).

(3) V. N. Ipatieff, H. Pines and R. C. Olberg, THIS JOURNAL, **70**, 2123 (1948)

(3a) This conclusion is based on the observation that during the reaction of *p*-cymene with cyclohexene the carbon atom ortho to the methyl group is substituted[1] and that the reaction between benzene and isomeric methylcyclohexenes, including 4-methylcyclohexene results in the formation of 1-methyl-1-phenylcyclohexane (V. N. Ipatieff, E. E. Meisinger and H. Pines, unpublished work).