80° and the heating continued until the evolution of nitrogen ceased. A considerable amount of brownish resinous material separated at this stage.

The mixture was repeatedly extracted with ether (500 ml.). The ether solution was treated with aqueous sodium bicarbonate to neutralize the acetic acid. The ether extract was dried over anhydrous magnesium sulfate and the ether distilled. The reddish oily residue was distilled at 1 mm. and 0.35 g. of a light brown semi-solid was obtained; the rest was non-distillable. The semi-solid material was subjected to vacuum sublimation and 0.2 g. of a slightly waxy solid resulted. On recrystallization from toluene, 100 mg. of pure 2-methoxy-5-hydroxypyridine was obtained; m. p. 81°.

Anal. Calcd. for $C_6H_7O_2N$: C, 57.6; H, 5.64; N, 11.2. Found: C, 57.79; H, 5.66; N, 11.06.

2,5-Dihydroxypyridine (5-Hydroxy-2-pyridone).—A solution of 90 mg. of 2-methoxy-5-hydroxypyridine in 1 ml. of hydrobromic acid (sp. gr. 1.5) in an atmosphere of nitrogen was refluxed for four hours. The hydrobromic acid was then removed *in vacuo* and this residue carefully neutralized with dilute aqueous sodium carbonate keeping the end-point slightly on the acidic side. The solution was then evaporated to dryness over a steam-bath in a current of nitrogen. The residue was sublimed at 1 mm. pressure. The sublimation started at 150° and the temperature was gradually raised to 180° during three hours. A pale yellow solid was obtained, weighing 70 mg. On recrystallization from ethanol, it was obtained as clusters of thin colorless needles which darkened at 215° and decomposed between 240–250°. Additional recrystallizations gave product showing the same characteristics upon heating.

Anal. Calcd. for $C_5H_5O_2N$: C, 54.01; H, 4.54; N, 12.6. Found: C, 54.10; H, 4.46; N, 12.41.

Summary

1. 2,5-Dihydroxypyridine (5-hydroxy-2-pyridone) has been synthesized by the replacement of the amino group in 2-methoxy-5-aminopyridine by hydroxyl and hydrolysis of the product.

2. This compound was not identical with the dihydroxypyridine obtained by pyrolysis of leucenol.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Preparation of Highly Purified Mustard Gas and its Action on Yeast[1]

BY VINCENT DU VIGNEAUD AND CARL M. STEVENS[2]

In undertaking a study of the biochemistry of mustard-type vesicants[3] it was of importance to have available the highly purified compounds. Even though the vesicant activity of impure preparations could be shown to reside solely in the β-chloroethyl sulfide, there could be no assurance that another physiological property of the material might not be due, in part at least, to impurities. That active impurities are indeed present in certain preparations of mustard gas (H) was shown clearly by Hellerman[4] who reported that H prepared by the Levinstein process[5] rapidly "covered" the *a* sulfhydryl groups of urease, whereas H prepared from thiodiglycol did not enter into rapid reaction with these groups.

In this Laboratory, we had begun a study of the effect of H-type vesicants on the growth of yeast[6] in a chemically defined medium.[7] We had found that yeast growth was inhibited by the addition of H to the medium. Of further importance was the observation that aqueous solutions which had been allowed to stand for several hours and which did not contain a detectable amount of bis-(β-chloroethyl) sulfide were also active in inhibiting the growth of yeast. The degree of inhibition by aged aqueous solutions varied greatly with different samples of H. Furthermore, the inhibitory action of the aged solutions was largely removed by heating the solutions in an open flask at 100°, or by extracting the solutions with petroleum ether.

When the yeast growth data indicated conclusively that there was a physiologically active impurity (or impurities) in the purest available samples of H, we undertook the purification of the vesicant. Despite its low melting point (14°), crystallization from a suitable solvent seemed the most promising method. It was found that crystallization of the compound occurred readily from dilute solution in petroleum ether or absolute ethanol if the solutions were cooled to approximately −75°. In this way purification was readily effected. Freshly prepared aqueous solutions of the recrystallized compound inhibited yeast growth to an extent similar to that observed with the unrecrystallized compound, whereas aged solutions of recrystallized H showed no evidence of any impurity inhibiting yeast growth. It thus appears that bis-(β-chloroethyl) sulfide is itself growth inhibitory, but that other inhibitors contaminating the redistilled samples of H were removed by the recrystallization. The recrystallized material also showed a somewhat higher melting point (14.5°) than that of the purest

(1) The work described in this paper was carried out under Contract OEMsr-144 between the Office of Scientific Research and Development and Cornell University Medical College and is described in Progress Reports to Section B4C, January, 1942, to June, 1942.

(2) Present address: Department of Chemistry, State College of Washington, Pullman, Wash.

(3) We have considered that vesicant compounds of the type R—SCH_2CH_2Cl (where R is an alkyl or aryl group) may safely be considered as analogous to mustard gas (bis-(β-chloroethyl) sulfide or H) in mode of action. For convenience, therefore, we shall refer to these compounds as "mustard-type vesicants" or "H-type vesicants."

(4) L. Hellerman, final summarization of NDRC work, Contract OEMsr-94.

(5) Felsing and Arenson, *Ind. Eng. Chem.*, **12**, 1065 (1920).

(6) Magne and Remy (*Bull. soc. chim. biol.*, **19**, 1092 (1937)) have reported some effects of mustard gas on yeast. See also, Herriott, Anson, and Northrop (*J. Gen. Physiol.*, **30**, 185 (1946)) for some recent studies.

(7) Snell, Eakin and Williams, THIS JOURNAL, **62**, 175 (1940).

samples obtained by us which had been prepared by redistillation.[8]

While the recrystallized H appeared to be stable for many weeks when stored at 5°, it underwent some change when heated at 85° for twelve hours, for the resulting material contained a yeast-growth inhibitor similar, at least in some properties, to the inhibitor in the original H.

The substance which inhibits yeast growth is neither 1,4-dithiane nor ethylene dichloride although these are known[9] to be formed in large amounts when ordinary H is heated to high temperatures, and we have found that dithiane is also formed from recrystallized H. Recently, Fuson and co-workers[8] have demonstrated that H is converted by heat into still other compounds. Further studies on the nature of the physiologically active substance would be of some interest, particularly as it appears to be present in very small amounts in the materials examined and therefore may actually be extremely toxic.

Experimental

Recrystallization of Mustard Gas.—The entire process was carried out in a cold room (5°). In a typical experiment, the starting material used was a sample of H prepared from thiodiglycol and hydrochloric acid. It had been carefully redistilled and possessed a melting point of 14.1–14.3°. Thirteen grams of the compound was dissolved in 200 cc. of absolute ethanol in a stoppered Erlenmeyer flask and the solution was cooled in an acetone–Dry Ice-bath (−75 to −80°) for about thirty minutes. The supernatant liquid was then decanted from the resulting crystals (or withdrawn through a sintered glass filter stick) as carefully and as completely as possible. The crystals were dissolved in a second 200-cc. portion of absolute ethanol and the procedure was repeated as many times as desired. Finally, the material was recrystallized by the same technique from 200 cc. of purified petroleum ether (b. p. 30–40°).[10] The compound separated in large crystals. The supernatant liquid was decanted and the remaining solvent was removed completely *in vacuo* at 5°. After three recrystallizations from alcohol and one from petroleum ether, the product weighed 7.5 g., m. p. 14.5°.

Measurement of Yeast Growth.—The yeast was Fleischmann Strain 139 of distiller's top yeast. Yeast growth was determined essentially by the method of Snell, Eakin and Williams,[7] with slight modification.[11] A small inoculum (equivalent to 0.06 mg. of dry yeast) of a 24-hour yeast culture was added to 10 cc. of medium in an open 50-cc. Erlenmeyer flask and the preparation was incubated for sixteen hours at 30°. Growth was measured by determination of the turbidity of the yeast suspension in a Klett–Summerson photoelectric colorimeter. In all cases, the *p*H of the cultures was maintained in the range 3.8 to 4.6 by addition of 1 cc. of 0.5 *M* citrate buffer (*p*H 4.6).

In Table I are recorded a few typical data on the effect of the various materials on yeast growth, the growth being expressed as per cent. of the growth in control flasks run concurrently to which no compound had been added. In control cultures the growth amounted to approximately 20 mg. of dry yeast.

TABLE I

EFFECTS OF VARIOUS SUBSTANCES ON YEAST GROWTH

Substance added	Concentration, mg./cc.	Yeast growth, % of control
Redistilled mustard gas (TMG)[a]	0.05	50
Redistilled mustard gas (TMG)	.1	20
Redistilled mustard gas (TMG)	.2	0
Redistilled mustard gas (LMG)[b]	.005	10
Redistilled mustard gas (LMG)	.015	0
"Aged" solution[c] of TMG	.5	70
"Aged" solution of TMG	1.0	50
"Aged" solution of LMG	0.0025	30
"Aged" solution of LMG	.005	10
"Aged" solution of twice-recrystallized[d] TMG	1.0	90
"Aged" solution of twice-recrystallized TMG	2.0	80
Thiodiglycol (Eastman)	5.0	40
Thiodiglycol (Eastman)	10.0	25
Recrystallized thiodiglycol[e]	5.0	90
Recrystallized thiodiglycol	10.0	75

[a] Material prepared from thiodiglycol and hydrochloric acid. [b] Material prepared by Levinstein process. [c] An aqueous solution of the material was allowed to stand for several hours at room temperature. [d] Material which had been recrystallized four times and then sublimed at 10° (0.1 mm.) showed an identical effect on yeast growth. [e] Thiodiglycol (Eastman Kodak Co.) was twice recrystallized by the general technique described previously for mustard gas using ethyl acetate as the solvent.

Summary

1. Mustard gas (bis-(β-chloroethyl) sulfide) can be readily recrystallized from absolute ethanol or petroleum ether at low temperatures.

2. All samples of mustard gas purified by redistillation were found to contain impurities inhibiting yeast growth. These impurities can be removed by recrystallization.

3. When heated, the recrystallized mustard gas again develops impurities inhibiting yeast growth.

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(8) The melting point of pure mustard gas has been variously reported; Mumford and Phillips (*J. Chem. Soc.*, 155 (1928)) found a value of 14.4°; Fuson, Lipscomb, McCusick and Reed (*J. Org. Chem.*, **11**, 513 (1946)) reported a value of 14.5°. Numerous studies have been reported on the purification of crude mustard gas, including a study of partial purification by crystallization from hydrocarbon solvents (Thompson and Odeen, *Ind. Eng. Chem.*, **12**, 1057 (1920)).

(9) Bell, Bennett, and Hock, *J. Chem. Soc.*, 1803 (1927).

(10) Commercial Skellysolve A was purified by shaking it with concentrated sulfuric acid, washing with water, drying, and distilling. This was found to remove a yeast growth inhibitor present in the original Skellysolve A.

(11) du Vigneaud, McKennis, Simmonds, Dittmer and Brown, *J. Biol. Chem.*, **159**, 385 (1945).