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ISOLATION OF UROPORPHYRIN III FROM CLOSTRIDIUM THERMOACETICUM

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SUMMARY. Clostridium thermoaceticum contains a porphyrin which, based on visible absorption and ^1H nmr spectra as well as on chromatographic behavior, has been identified as uroporphyrin III.

INTRODUCTION

Clostridium thermoaceticum synthesizes acetate from CO_2 via a corrinoid-dependent pathway (1,2). It also synthesizes corrinoids, which is evidenced by the facts that the organism does not exhibit a corrinoid requirement that between 30 and 70 μmoles of a mixture of corrinoids can be isolated from 100 g of wet cells (3), and that radioactive δ-aminolevulinic acid is incorporated into the corrinoids (4). Over 20 different corrinoids have been isolated and characterized from C. thermoaceticum (5,6). They include Co-methylcobyric acid and Coα-(5-methoxy-benzimidazolyl)-Coβ-methylcobamide, which function in the acetate synthesis (1) and, in addition, di-, tri-, tetra-, and pentamides of cobyrinic acid, cobyric acid, cobinamide, cobinamide phosphate, and cobinamide guanosine diphosphate, which are all considered to be intermediates in the synthesis of cobamides from cobyrinic acid (7). The biosynthesis of 5-methoxybenzimidazole has been studied in C. thermoaceticum (8), and the presence of δ-aminolevulinate dehydratase has been established (9). Besides these observations little is known about the biosynthesis of corrinoids in C. thermoaceticum. In this report we describe the isolation of uroporphyrin III, which is the oxidation product of uroporphyrinogen III. The latter is considered

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an intermediate in the biosynthesis of tetrapyrroles from δ-aminolevulinate and is at the branching point for the formation of porphyrins and corrinoids (10,11).

MATERIALS AND METHODS

C. thermoaceticum (DSM 521) was grown as previously described (12). Uroporphyrin I and III octamethyl esters were obtained from Porphyrin Products, Logan, Utah, U.S.A. UV and visible spectra were recorded with a Beckman Acta CV spectrophotometer and proton nuclear magnetic resonance spectra with a JEOL-PFT-100 one hundred MHz nmr spectrometer in the Fourier transform mode. $CDCl_3$ was purchased from Aldrich Chem. Comp. Chromatography on paper of free uroporphyrins was done according to With (13) and of uroporphyrin octamethyl esters, according to Falk and Benson (14).

Isolation of porphyrin from C. thermoaceticum. 100 g of wet or frozen cells were suspended in 300 ml of 50 mM Tris.HCl, pH 7.6. The suspension, kept below 5°C, was passed twice through a Manton-Gaulin homogenizer. An equal volume of pre-cooled (below -10°C) acetone was added to the homogenate. After removal of undissolved and precipitated material by centrifugation the clear amber-colored solution was applied to a Whatman DE-23 column (2.5x20 cm) previously equilibrated with 50 mM Tris.HCl, pH 7.6. A red material, which was identified as a mixture of corrinoids, passed directly through the column, however, a brownish band containing rubredoxins, ferredoxin and a porphyrin was formed on top of the column. The column was washed with 300 ml of the Tris.HCl buffer and then the rubredoxins and the ferredoxin were eluted using a linear gradient consisting of 500 ml 0.07 M NaCl and 500 ml 0.7 M NaCl, both containing 50 mM Tris.HCl, pH 7.6. After elution of the ferredoxin, a pink-colored band containing a porphyrin remained on the column. It was eluted by a solution of 2 M NaCl in 50 mM Tris.HCl, pH 7.6.

Esterification of the porphyrin (15). The pink eluate containing the porphyrin from the DE-23 column was evaporated to dryness in a flash-evaporator at 60°C. From the resulting powder, the porphyrin was extracted with 100 ml of a methanol/H_2SO_4 (95/5, vol/vol) solution. This solution was then kept in the dark at 4°C for 24 h to complete the esterification. An equal volume of ice chips was added to the esterification mixture from which the porphyrin methyl ester was extracted using 100 ml of chloroform. The chloroform solution was washed first with water, then 2 N NH_4OH and, finally, with water until the pH of the water wash was neutral. The chloroform was then removed by evaporation and the porphyrin methyl ester was dissolved in 5 ml of chloroform/methanol (1/1,vol/vol). From this solution when stored at 4°C, the porphyrin methyl ester crystallized.

RESULTS.

Light absorption spectra. The positions of absorption maxima of the isolated porphyrin in Tris.HCl, pH 7.6, and 0.5 N HCl, and of the porphyrin methyl ester in chloroform are listed in Table I. The spectrum in Tris.HCl is four-banded in the visible region and has a Soret band at 395 nm. In HCl, the visible spectrum is two-banded and the Soret peak is at 406 nm. The spectrum of the porphyrin methyl ester is similar to the "neutral" spectrum of the porphyrin with four bands and a Soret peak at 406 nm. The spectra are typical for

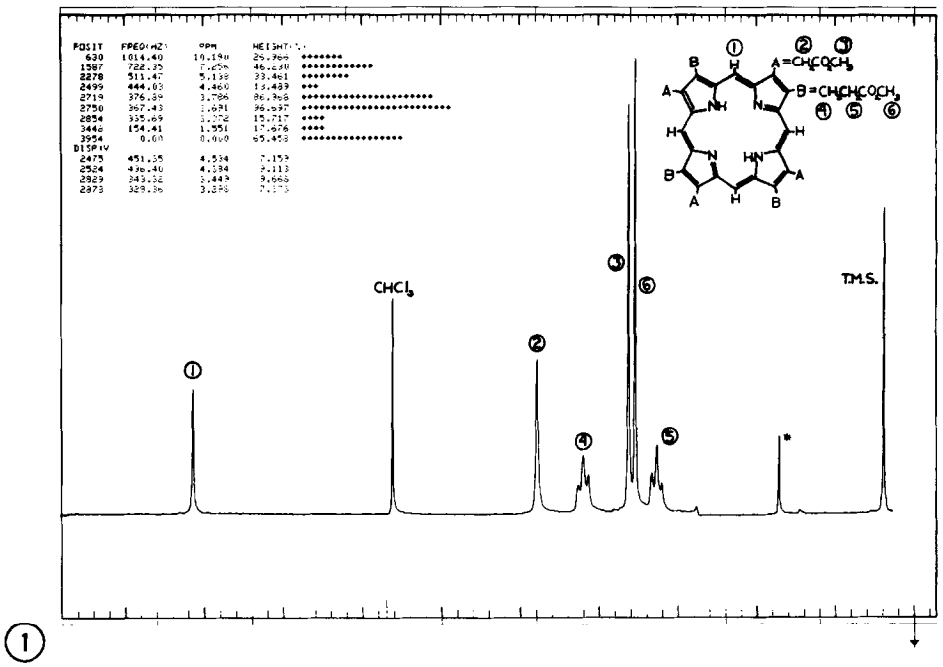
Table I. Light absorption spectra of isolated porphyrin.

Compound and solvent	Absorption maxima (nm)[a]				
Porphyrin in Tris, pH 7.6	610	559	536	500	395
	(0.60)	(1)	(1.30)	(1.72)	(27.3)
Porphyrin in 0.5M HCl	594	552			406
	(0.36)	(1)			(30.0)
Porphyrin methyl ester in $CHCl_3$	626	572	536	502	406
	(0.60)	(1)	(1.32)	(2.28)	(31.2)

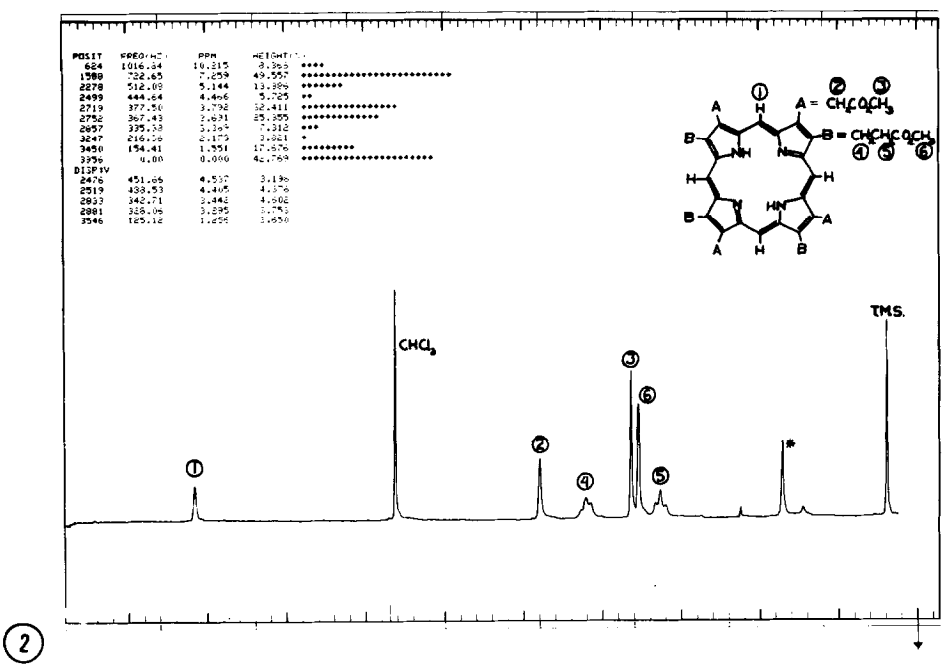
[a]The values within parentheses are ratios of absorbancies at wavelengths indicated.

porphyrins which do not contain a metal. The positions of the absorption maxima as well as the relative absorbancies are close, if not identical, to those obtained for uroporphyrin I and III by Mauzerall (16) and Borgorad (17). The commerical samples of the octamethyl esters of uroporphyrin I and III also gave the same spectra as the isolated porphyrin methyl ester. The porphyrin in Tris.HCl was reduced by sodium borohydride but not by dithionite, which was demonstrated by an almost complete disappearance of the visible absorption bands. On oxidation, these bands reappeared. The spectral properties of the isolated porphyrin strongly indicate that it is a uroporphyrin. Using a molar extinction coefficient of 2.15×10^5 at 406 nm for the uroporphyrin octamethyl ester (16), it was found that about 2 μmoles of uroporphyrin was normally obtained from 100 g of wet cells of C. thermoaceticum.

Paper chromatography. A R_f value of 0.78 was obtained during paper chromatography of the free isolated porphyrin in a solvent consisting of ammonia and lithium chloride (13). This high R_f value indicates a porphyrin containing at least 7, but more likely 8, carboxyl groups as in uroporphyrin. The methylated porphyrin was chromatographed on paper in a dioxan-kerosene mixture together with the commercial samples of uroporphyrin I and III methyl esters (14). R_f values ranging from 0.11 to 0.19 were obtained for the uroporphyrin I octamethyl



(1)



(2)

Fig. 1. ^{1}H nmr spectrum of uroporphyrin I octamethyl ester in $CDCl_3$. The peak marked * is an impurity in the $CDCl_3$ that was used. The spectrum represent the accumulations of 120 scans.

Fig. 2. ^{1}H nmr spectrum of uroporphyrin III octamethylester in $CDCl_3$. The spectrum represents the accumulations of 100 scans.

ester, whereas R_f values from 0.74 to 0.78 were found for the uroporphyrin III octamethyl ester and the isolated porphyrin methyl ester.

Use of ^{1}H nmr to distinguish between uroporphyrin I and uroporphyrin III octamethyl esters. ^{1}H nmr spectra with peak assignment of commercial samples of uroporphyrin I octamethyl ester and uroporphyrin III octamethyl ester dissolved in $CDCl_3$ are shown in Fig. 1 and 2, respectively. The corresponding spectrum of the isolated porphyrin methyl ester is given in Fig. 3. The three spectra are nearly-identical, which indicates a close similarity in structure of the three compounds. In the uroporphyrin I octamethyl ester, the acetate and propionate ester functions are symmetrically placed around the porphyrin ring. Since in the uroporphyrin III octamethyl ester the placement of the acetate and propionate side chains are reversed on one of the pyrrole rings (ring D) this compound does not have the symmetry of the uroporphyrin I octamethyl ester. This is reflected in broadened and assymetrical nmr signals, which is illustrated

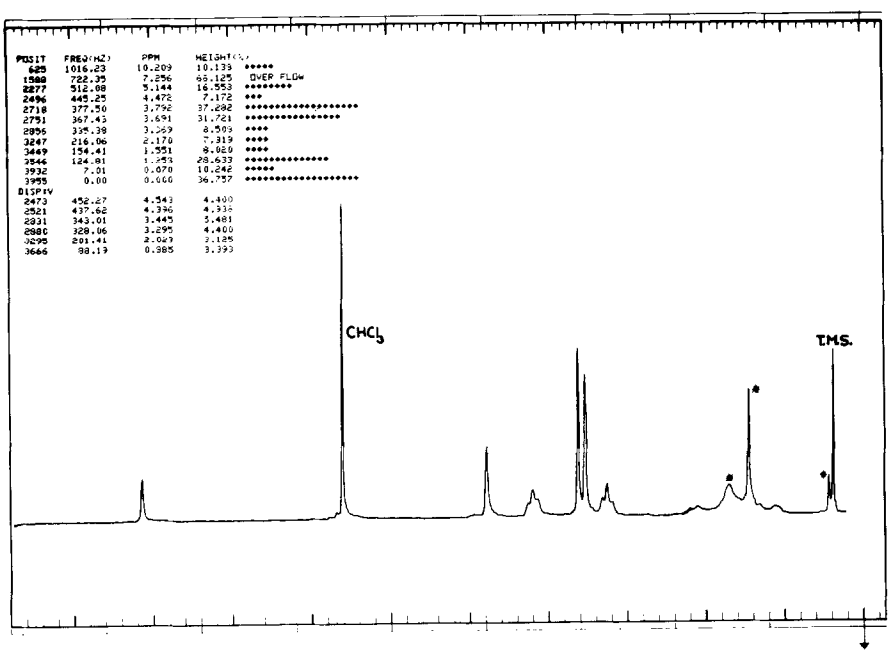


Fig. 3. ^{1}H nmr spectrum of porphyrin methyl ester isolated from C. thermoaceticum. * Indicates impurities in $CDCl_3$ that was used and of stop-cock grease (Lubriseal, Arthur H. Thomas Comp.) in the sample. The spectrum represents the accumulations of 410 scans.

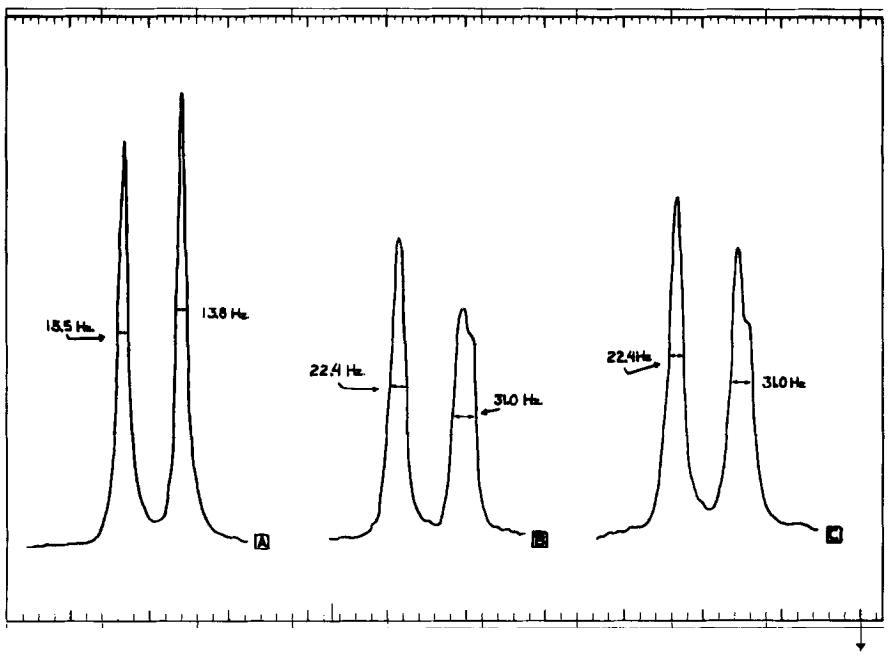


Fig. 4. Expansion of ^{1}H nmr signals of terminal methyl ester groups at 3.79 and 3.69 ppm, showing widths at half heights of the peaks. Curve A = uroporphyrin I octamethyl ester. Curve B = uroporphyrin III octamethyl ester. Curve C = porphyrin methyl ester from C. thermoaceticum.

in Fig. 4. In this figure the signals of the methyl groups of the propionate and acetate esters are expanded. The signals (curve A) from uroporphyrin I octamethyl ester are sharp and symmetrical, whereas corresponding signals (curve B) from the uroporphyrin III octamethyl ester are broader and assymetrical. This is best appreciated by comparing the width at half heights of the signals, as shown in Fig. 4. The porphyrin methyl ester isolated from C. thermoaceticum exhibits signals (Curve C) identical to those of the uroporphyrin III octamethyl ester.

DISCUSSION.

About 2 μmoles of a porphyrin has been isolated from 100 g of wet cells of C. thermoaceticum. This porphyrin based on visible absorption and ^{1}H nmr spectral properties as well as on chromatographic behavior has been identified as uroporphyrin III. Uroporphyrin III is the oxidation product of uroporphyrinogen III, which is considered an intermediate in porphyrin and corrinoid biosynthesis

(7,10,11). A cytochrome b has been found in C. thermoaceticum (18) however, this microorganism predominantly synthesizes corrinoids (5,6). The demonstration and isolation of uroporphyrin III from C. thermoaceticum appears to be in agreement with the concept that uroporphyrinogen III is an intermediate in corrinoid biosynthesis.

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