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A new cyclopyrophosphate as a bacterial antistressor?

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In a number of bacteria an unusual glycosyl pyrophosphate (^{31}P NMR signal chemical shift at about −15 ppm) was detected when the cells were subjected to oxidative stress. This substance from *Brevibacterium ammoniagenes* has now been identified as 2-methyl-butan-1,2,3,4-tetraol-2,4-cyclopyrophosphate, which is accumulated in the cell under certain conditions in concentrations of about 50 mM. It is now suggested that this compound is the long sought after bacterial antistressor.

Oxidative stress; Bacterium; Cyclopyrophosphate

1. INTRODUCTION

It is widely believed that oxidative stress of cells is involved in a number of pathologies [1]. Bacteria provide a good model for this process and have been shown to respond to chemically induced oxidative stress with the biosynthesis of at least a dozen specific proteins and micromolar amounts of dinucleotidepolyphosphates which are sometimes termed alarmons [2]. An unusual organic pyrophosphate (2,3-cyclo pyrophosphoglycerate) accumulated in *Methanobacterium thermoautotrophicum* in substantial amounts but no relation to oxidative stress was reported [3]. Recently, we have observed in *Micrococcus luteus* and *Brevibacterium ammoniagenes* another unusual pyrophosphate (^{31}P NMR signal chemical shifts of −14.8 and −10.7 ppm) which was synthesized in response to oxidative stress induced with benzylviologen, menadione and other redoxcycling agents [4]. We have now isolated and purified this substance, which can be accumulated in concentrations up to 50 mM in the *B. ammoniagenes* cytoplasm, and determined its chemical structure.

2. EXPERIMENTAL

Brevibacterium ammoniagenes ATCC 6872 was cultivated in rotating flasks in a peptone-NaCl-yeast extract medium at 30°C, and benzylviologen chloride (Reachim) was added to 50 μg/ml at the end of logarithmic growth for induction of synthesis of the new compound. The compound was extracted and purified as described [4,5]. Molecular mass spectra were registered on an MI-1201 E Soviet instrument, and NMR spectroscopy was performed on Bruker AM-300, AM-400 and WH-250 instruments equipped for proton, carbon-13 and phosphorus-31 measurements using standard programs.

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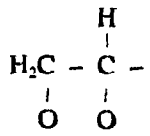
3. RESULTS AND DISCUSSION

As determined from ^{13}C NMR spectra and ^{1}H NMR data (Figs. 1 and 2 and Table I) the substance under study consists of five C atoms:

(i) $CH_3-\underset{|}{\overset{|}{C}}-$ (σ = 17.8 and σ = 85.3 ppm);

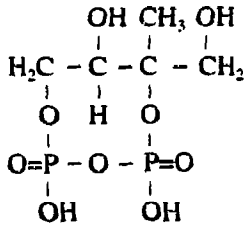
(ii) an isolated methylene group (σ_C = 68.4; σ_{Ha} = 3.58 and σ_{Hb} = 3.74 ppm for solution in D_2O);

(iii) a three-proton system,



two of which are certainly attached to the tertiary carbon atom.

The NMR data therefore are consistent with the structure of 2-methylbutan-1,2,3,4-tetraol-2,4-cyclopyrophosphate and also the ion signal, *m/e* = 278, determined by fast atom bombardment mass spectrometry [5,6]:



The protein synthesis inhibitor, chloramphenicol (50 μg/ml), was found not to prevent the accumulation of this substance. After transfer of the bacterial cells to a

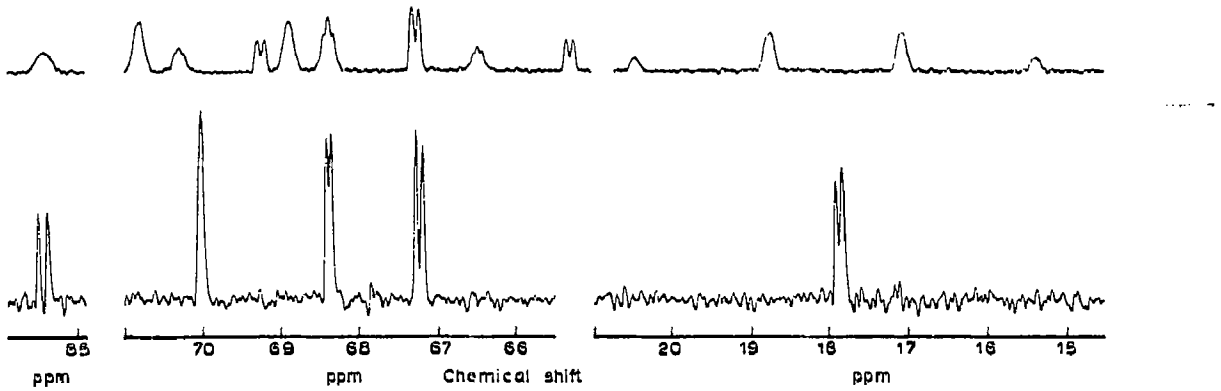


Fig. 1. ^{13}C NMR spectra in D_2O of a new pyrophosphate isolated from *Brevibacterium ammoniagenes*. (Lower trace) proton decoupled spectrum (line splitting is due to ^{13}C–^{31}P spin–spin coupling); (Upper trace) proton coupled spectrum (methanol as standard, σ = 50, 15 ppm).

fresh, benzylviologen-free medium the new pyrophosphate gradually disappeared; this suggest that it most likely is involved in cell repair processes.

Degradation products of this pyrophosphate or its immediate precursors are not known but one can propose that the pyrophosphate is synthesized via a condensation of two phosphoenolpyruvate molecules, where one –COOH is removed while the other is reduced to a $-CH_2OH$ group. A pathway via phosphomevalonyl pyrophosphate is also possible.

How can this substance be thought to help the cell to resist oxidative stress?

Firstly, the conserve pyrophosphate could energy, phosphorous and carbohydrates during the period of disorganization of the cell metabolism.

Secondly, a carbohydrate portion of the molecule may participate in the interception of poisonous free radicals. (It is worthwhile to point out that both free hydroxyl groups are facing the same side of the molecule, because borate ions have been shown to form a complex which produces a strong up field shift of ^{31}P NMR resonance from −14.8 to −15.5 ppm.) Also, the phosphate portion of the molecule may be involved in the chelation of the cations responsible for the propagation of the oxidative damage. This last suggestion is not simply speculation because the substance does strongly bind Fe^{2+} and Cu^{2+} ions, and, what is of special interest,

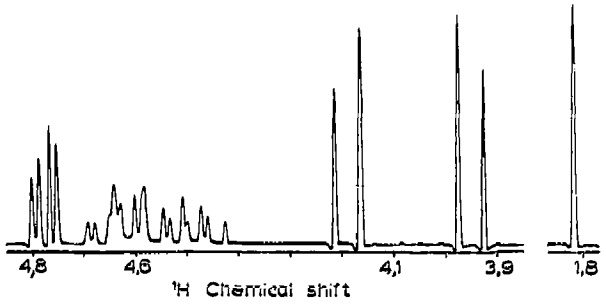


Fig. 2. ^1H NMR spectrum of a new pyrophosphate isolated from *Brevibacterium ammoniagenes*, in pyridine-d_5 with tetramethylsylane as a standard (0 ppm).

its phosphate groups reveal binding selectivity: the σ = −14.8 ppm phosphate binds Mg^{2+} and Ca^{2+} while the σ = −10.7 ppm phosphate interacts preferentially with Zn^{2+} and Cd^{2+} ions.

Thirdly, energy released by the pyrophosphate hydrolysis may be used to form conjugates with some toxic substances.

Additionally, since the operation of protein synthesis machinery is not necessary for the accumulation of this substance it is a good compound for a fast response to the stress.

Several years ago we noticed a signal of σ = −15 ppm in ^{31}P NMR spectrum of *Mycobacterium smegmatis* extracts, and later also observed the same in *M. phlei* [7]. It now appears that this signal is due to organic pyrophosphate present normally in mycobacteria and that this substance is partly responsible for the well-known tolerance of *Mycobacterium* (i.e. *M. tuberculosis*, *M. leprae*) to host immune system attacks, including oxidative stress.

Recently it was reported that a substance, possibly identical to that discussed above, had been isolated from a strain of *Desulfovibrio desulfuricans* [8]. Although a molecular formula of the substance was not determined, the NMR results coincide with those for 2-methylbutan-1,2,3,4-tetraol-2,4-cyclopyrophosphate. The biological functions of this substance may be even broader than we expected initially, and may be important not only in resistance to oxygene per se but also

Table I

^1H NMR spectra parameters of a newly isolated compound from *Brevibacterium ammoniagenes* in pyridine-d_5.

Proton	Chemical shift (ppm)	Spin coupling constant
1	3.88	$J_{1,1'}$ 11.7; $J_{1,2'}$ <0.5
1′	4.12	$J_{1',2'}$ <1
2′	1.82	$J_{2',3}$ <0.5
3	4.90	$J_{3,4}$ 3.4; $J_{3,4'}$ 8.5
4	4.75	$J_{4,4'}$ 11.5; $J_{4,P}$ 18.0
4′	4.60	$J_{4',P}$ 10.0

in the metabolic control of deleterious effects of any oxidant, or the products of partial reduction of sulfate, which induce a perturbation of the redox status of cells comparable with that of oxidative stress.

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