ε-N-TRIMETHYL-L-δ-HYDROXYLYSINE PHOSPHATE AND ITS NONPHOSPHORYLATED COMPOUND IN DIATOM CELL WALLS

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SUMMARY. O-Phosphoester of ε-N-trimethyl-L-δ-hydroxylysine and its nonphosphorylated compound were found in the cell walls of six species of diatoms and were isolated from the proteinaceous material of Navicula pelliculosa. Final evidence for the chemical structure was obtained by comparison with the synthetic nonphosphorylated compound.

In a previous paper (1) we reported the discovery of 3, 4-dihydroxy-L-proline in the proteinaceous material of diatom cell walls. Two dimensional paper chromatography (2) revealed, in addition, the presence of a number of unidentified ninhydrin-reacting compounds. This paper describes the identification of two of these compounds, ε-N-trimethyl-L-δ-hydroxylysine phosphate (THLP, spot 5 Fig. 1 and Fig. 2A), and ε-N-trimethyl-L-δ-hydroxylysine (THL, spot 1 Fig. 1 and Fig. 2B). These two are of interest since they extend the family of known lysines.

For the isolation of THLP, acid hydrolyzate (1) obtained from 150 g of cell walls (prepared from 6,800 g of wet cells), was passed through a Dowex AG 50W-X4-ion-exchange column (H^+ form, 10 to 50 mesh, 4 x 65 cm) and eluted with 2N aqueous ammonia. The eluate was concentrated at reduced pressure, and the residue, dissolved in aqueous solution of 0.3% pyridine-0.03% acetic acid, (Solution I), was passed through Amberlite CG-50 column (pyridinium form, 200 to 400 mesh, 2.4 x 90 cm) at 45° C. The column was eluted stepwise, with solutions I, (600 ml), aqueous solutions of 2% pyridine-0.2% acetic acid (Solution II; 1300 ml), and 3% pyridine-0.3% acetic acid (Solution III; 900 ml). Acidic and neutral amino acids were eluted by solution I (Fraction I), basic amino acids were eluted by solution II (Fraction II) and contained lysine, spots 1, 2, 11, 12 and 36; arginine was eluted by solution III. Fraction I was concentrated at reduced pressure and passed through Dowex AG2-X8 column (acetate form, 200 to 400 mesh, 2.4 x 90 cm) at 45° C; the column was washed with water and then with 0.1N acetic acid. The course of the elution was followed by thin-layer chromatography and ninhydrin. The fractions containing THLP, which included small amounts of neutral amino acids, were combined and concentrated. The residue was dissolved in 0.1N acetic acid, passed through Sephadex G-15 column (40 to 120μ, 2.4 x 190 cm), and eluted with 0.1N acetic acid (800 ml). THLP was obtained as a single peak at 320 ml.

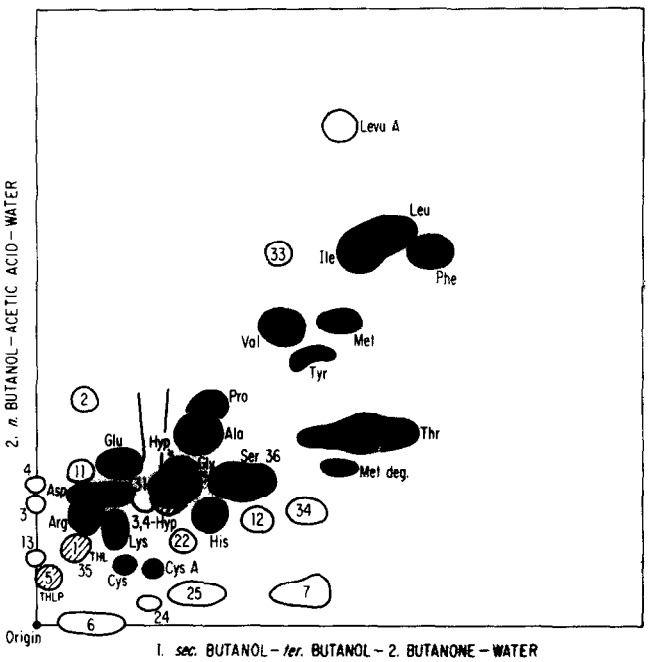
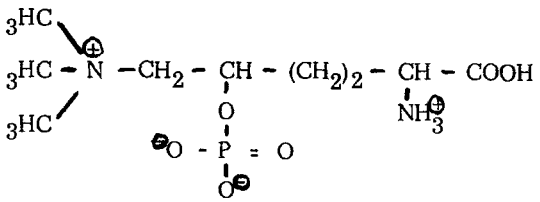
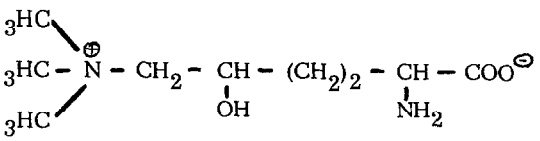


Fig. 1. Tracing of a two-dimensional ascending paper chromatogram of amino acid hydrolysate of cell walls of N. pelliculosa on Whatman 3HR paper. The chromatogram was sprayed with ninhydrin and developed at room temperature until spots appeared. It was then heated at 80° C for 5-10 min, and continued at 110° C for 10 min. The following spots appeared at 110° C: levulinic acid (Levu. A), 6, 7, 34; 31 after intensive heating. Met deg. = degradation product of methionine.



A



B

Fig. 2. A. ϵ-N-Trimethyl-δ -hydroxylysine phosphate.
B. ϵ-N-Trimethyl-δ -hydroxylysine.

After lyophilization, it yielded 300 mg of an hygroscopic light-brown powder, which melted at 60 to 130° C with decomposition. Attempts to crystalize the compound in ethanol-water, and acetone-water yielded a syrupy material.

The specific rotations $[\alpha]_D^{25}$ (measured with a Durrum-Jasco Optical Rotary Dispersion Recorder Model ORD/UV-5) were +30.0° and +50.0° at concentrations of 0.5 percent in H_2O and in 2N HCl, respectively. As judged by conformity to the rule of Lutz-Jirgensons (3), the compound has L configuration. Analysis showed the following values for $C_9H_{21}O_6N_2P \cdot 1/2H_2O$: C, 36.56; H, 7.22; N, 9.01; and P, 10.30 percent; calculated values were: C, 36.86; H, 7.56; N, 9.55; and P, 10.56 percent. The barium salt was a hygroscopic white powder, and the analysis showed the values for $C_9H_{20}O_6N_2P \cdot 1/2Ba \cdot H_2O$ to be: C, 30.0; H, 6.0; N, 7.4; P, 8.2; and Ba, 18.2 percent; calculated values were: C, 29.22; H, 5.99; N, 7.57; P, 8.37; and Ba, 18.57 percent. Infrared spectrum (measured with a Perkin-Elmer 237B Grating Infrared Spectrophotometer) showed γmax at 3425 (NH, OH, CH); 1625 ($COO^{\ominus}$), 1490 (NH_3^+), 1353, 1190 (P-O) and 1080 (P-O-C) cm^{-1}. The chromatographic and electrophoretic migrations, the elution time in the amino acid analyzer, and reactions to various reagents are given in Table 1. The compound did not react with ninhydrin after treatment with nitrous acid (4).

For the isolation of THL, the concentrated fraction II was charged on Dowex AG 50W-x4 column (pyridinium form, 200 to 400 mesh, 2.4 x 87 cm) at 45° C, buffered with 0.2M solution of pyridine and formic acid (pH 3.1), and eluted with 1.0M solution of pyridine-acetic acid buffer (pH 5.0, 1500 ml) (5). The first peak (520 ml) consisted of THL and compound 12, followed by peaks 36, (590 ml), lysine (670 ml), 2 (700 ml) and ammonia. The THL peak was concentrated, and the residue was passed through a Dowex AG1-x8 column (OH^- form, 100-200 mesh, 1 x 20 cm) and eluted with water (450 ml). After the aqueous effluent was concentrated the residue gave a single spot on a two dimensional chromatogram. In the amino acid analyzer (6), however, it showed 3 peaks and NH_3; elution times were as follows: allo THL (ATHL), 197 min; THL, 200 min; NH_3, 260 min; and unknown, 209 min. For further purification, the iodide derivative was prepared by dissolving the residue in 0.5 ml of HI (47%) and drying it over KOH in a vacuum desiccator. The yellow residue was treated with small volume of absolute ethanol to remove soluble yellow material. The free THL was recovered by passing it through a small Dowex AG1-x8 column (OH^-form, 1 x 2 cm), yielding 26.5 mg of a gummy, slightly yellowish compound. It showed a single peak in the amino acid analyzer. The specific rotations $[\alpha]_D^{24}$ were +15.0° and +23.1° at concentrations of 0.84 percent in H_2O and 2N HCl, respectively; the compound has the L configuration (3).

The THL iodide consisted of hygroscopic, white microscopical needles, which melted at 243° C with decomposition. The specific rotation $[\alpha]_D^{25}$ was +45.0° at a concentration of 0.4 percent in H_2O. Analysis showed the values for $C_9H_{20}O_2N_2I_2$

(the formula is that of the lacton form as evident from the IR spectrum band at γ_{max} 1750 cm^{-1}.) were: C, 24.72; H, 4.32; N, 6.19; and I, 57.62 percent; calculated values were: C, 24.45; H, 4.56; N, 6.34; and I, 57.42 percent. The chromatographic properties of THL and reaction to various reagents are given in Table 1. Unlike THLP, THL did not react with ammonium molybdate.

TABLE 1

Comparison of Chromatographic and Electrophoretic Migrations, Elution Time and Chemical Reactions of ϵ-N-trimethyl-L-δ-hydroxylysine phosphate (THLP) and ϵ-N-trimethyl-L-δ-hydroxylysine (THL).

Procedure	THLP	THL
Thin layer chromatography R_F in solvents:*		
I	0.13	0.28
II	0.12	0.18
III	0.09	0.11
Electrophoretic mobility in solvents:**		
I pH 1.9	-2.4cm	-6.1cm
II pH 6.9	+2.6	-7.1
III pH 9.0	-	-12.7
Emergence of peak on amino acid analyzer:		
Dus system (6)	51 min	200 min
Hamilton system +	105 min	948 min
Color reactions:++		
Ninhydrin	blue-violet	blue-violet
Iodoplatinate	blue	blue
Dragendorff	orange	orange
Ammonium molybdate	blue	negative

* Chromatograms run on Eastman Chromatogram Cellulose Sheets (6064), without fluorescent indicator. Solvents contained: I. n butanol, acetic acid, water (12:3:5); II. n butanol, acetic acid, water (4:1:5); III. sec-butanol, tert-butanol, 2-butanone, water (4:4:8:5), 0.5 percent diethylamine.

** Carried out in the electrophoresis apparatus described by A.M. Crestfield and F.W. Allen, Anal. Chem., 27, 422 (1955). Whatman paper No. 1 was used and a voltage of 33.3 v/cm in solvents: I. 0.6N formic acid-2N acetic acid buffer (pH 1.9) for 20 min; II. 0.02M sodium phosphate buffer (pH 6.9) for 10 min; III. 0.05M borate buffer (pH 9.0) for 50 min.

\+ Kindly run by Dr. P.B. Hamilton (P.B. Hamilton Anal. Chem., 35, 2055 (1963)).

++ Ninhydrin solution 0.2% in n butanol. Iodoplatinate: J.V. Jackson and M.S. Moss, in Chromatographic and Electrophoretic Techniques, I. Smith, Ed. (Interscience New York, 1969), vol. 1, p. 519. Dragendorff: T. Kariyone and Y. Hashimoto, Yakugaku Zasshi, 71, 436 (1951). Ammonium molybdate, S. Burrows, F.S.M. Grylls, and J.S. Harrison, Nature, 170, 800 (1952).

THLP was hydrolyzed by alkaline phosphatase (E. C. 3. 1. 31) to THL and phosphoric acid in equal molar quantities. When THL was heated with 6N HCl for 72 hours at 105° C, it produced a mixture of two components, as observed in the amino acid analyzer, consisting of THL and ATHL (61:39). A similar hydrolysis of THLP yielded a mixture of the same two components and a trace amount of THLP.

These results suggested that THLP is the monophospho-O-ester of the hydroxy amino acid THL and is relatively stable to acid hydrolysis. This stability is similar to that of O-phosphocholine, O-phosphoethanolamine, O-phosphoserine and O-phosphohydroxylysine (7).

The positive color reaction of THLP and THL for tertiary and quaternary amines, (iodoplatinate and Dragendorff), the hydroscopic properties, and the failure to crystalize, suggest that the compounds are quaternary amines. This is further suggested by the nuclear magnetic resonance (NMR) spectra of both compounds, (obtained with a Varian HR-200 spectrometer in D_2O and dimethyl silapentane sulfonate as internal reference) which showed the identical signal for N-trimethyl group at 3. 18 ppm. Other NMR data and the elementary analysis indicated that the compounds are hydroxylysine derivatives.

Final evidence for the structure of THL was obtained by its synthesis from a DL (+allo)-δ -hydroxylysine using a method similar to that described by Takemoto et al., (8). In the amino acid analyzer the synthetic compound showed two peaks consisting of ϵ-N-trimethyl-DL-δ -hydroxylysine and its allo isomer; the peaks were identical to those of THL and ATHL, and both the R_F values and the electrophoretic migrations in the various solvents were identical to those of the natural THL.

The DL-compound, obtained as the iodide derivative as described for THL, consisted of hygroscopic white fine needles which melted at 231° C with decomposition. Analysis showed the values for $C_9H_{20}O_2N_2I_2$ were: C, 24. 61; H, 4. 65; N, 6. 32; and I, 57. 24 percent; calculated values were: C, 24. 45; H, 4. 56; N, 6. 34 and I, 57. 42 percent. IR spectrum of this compound was essentially identical to that of THL. The iodide-free compound showed a single peak in the amino acid analyzer identical to that of THL; the NMR spectra were also identical to that of THL.

The possibility that the THL in the hydrolysate of cell wall might have been derived from THLP during the hydrolysis had to be considered. However, the obtained amount of THL + ATHL in the hydrolysate exceeded by 30% the calculated amount to be obtained from the quantity of THLP found. The calculation was based on determination of THL + ATHL in hydrolysate of pure THLP in 6N HCl at 100° C in the amino acid analyzer. The result thus indicates that THL is present in the walls.

Analysis with paper chromatography (2) and the amino acid analyzer (1) showed that THL and THLP are present in acid hydrolyzates of the walls of five marine diatom species of differing morphological complexity: Nitzschia angularis, Nitzschia incerta (minute quantities), Cylindrotheca fusiformis, Cyclotella cryptica, and Phaeodactylum

tricornutum (fusiformis and oval) (9). It was not found in the pool of N. pelliculosa (extracted with cold 10% trichloroacetic acid).

Of the previously identified lysines, L-δ -hydroxylysine occurs in considerable quantities in collagen; O-phosphohydroxylysine has been reported in the cellular fluids of calf embryo muscle (7); and ε-N-trimethyl-lysine has recently been found in histones (10), in cytochrome c of wheat germ and Neurospora (11), and in skeletal myosin (12). Thus to the already-known hydroxylysines, THL and THLP add the trimethylated analogues.

Of particular interest is the fact that these lysines occur in the siliceous cell wall of the diatom. Although the nature of the silicifying matrix has not yet been elucidated, it is possible that the 3,4-hydroxyproline, the THL and the THLP all occur in the matrix. Collagen, which is distinguished in large part by the presence, among other amino acids, of 3- and 4-hydroxyproline and hydroxylysine, serves as the matrix for calcification. Thus it may be that the two types of matrices, although differing in many respects, contain analogues constituents.

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