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The Isolation of Protein-Bound Phosphorylmuramic Acid from *Lactobacillus casei*

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A new phosphorylated amino acid has been isolated from a hydrolysate of the protein residue of *L. casei*. It has been identified as phosphorylated muramic acid. The position of the phosphate group must probably be in the primary alcoholic group. The yield of crystalline material was about 150 mg from 1 kg fresh bacteria.

In earlier communications it was demonstrated that acid hydrolysates of protein from *L. casei* mainly contained a phosphorylated amino acid [1,2] which was not identical with the phosphorylated amino acids isolated with a similar procedure from animal proteins [3]. The amino acid contained one nitrogen atom per phosphoryl group and ionophoretically behaved as a neutral amino acid. The rate of ^{32}P-incorporation into this amino acid was compared with that of other phosphorus compounds such as free and RNA-bond nucleotides. By preparing large amounts of the phosphorylated compound it could be shown that it contained about 50 % oxygen and behaved as an amino sugar. Later, the dephosphorylated compound was found to be identical with 3-O-α-carboxyethyl-D-glucosamine [4] which also has been named muramic acid (Mur)*.

PREPARATION OF PHOSPHORYLMURAMIC ACID

Lactobacillus casei (7461) was cultivated in a medium with the following composition: 10 g of Dextropur **, 10 g of $NaOCOCH_3 \cdot 3\ H_2O$, 10 g of Bactopeptone ***, 1 g of yeast extract ****, 25.4 ml of liver extract §, 0.5 ml of

* The following abbreviations are used: Mur = muramic acid; MurP = O-phosphorylmuramic acid; SerP = O-phosphorylserine.

** Glucose manufactured by Corn Products Co., New York.

*** Parke, Davis and Co.

**** Difco Laboratories.

§ 100 g of Difco Liver was dissolved in 1 330 ml water and kept at 50°C for one hour and subsequently at 80° for 5 min. After filtration and concentration the filtrate was lyophilized This material was dissolved to contain 15 mg per ml.

salt mixture * per 1 000 ml of medium at pH 6.8. For large scale cultivation (90 l) the necessary ingredients were first dissolved in 5 l water and heated at 100°C for about 15 min. After cooling a dark precipitate was removed by filtration. The filtrate was evenly distributed in thirty 6 liter Erlenmayer flasks and subsequently water was added to a final volume of 3 l per flask. After adjusting the pH to 6.8 sterilization was accomplished by autoclaving the flasks 15 min at 118°C.

Inocula for large scale cultivations was prepared by transferring a small amount of growth from a stab culture [5] to two 10 ml flasks, each containing 5 ml of medium. The amount of inocula was increased in the four subsequent steps (2, 3, 4, and 5) according to Table 1. 300 ml of suspension from step

Table 1. Increase of inocula for large scale cultivation.

Step No.	Ml of inocula to ml of culture		Incubation time, h
1	0.1	2 × 5	24
2	8 × 0.5	8 × 5	24
3	30 × 1	30 × 20	17
4	30 × 20	30 × 100	17
5	30 × 100	30 × 300	17
6	30 × 300	30 × 3 000	

5 was then added to each 6 l flask containing 3 l sterilized medium. 1 mC radioactive phosphate per liter medium was added at the same time. Previously it had been shown that the presence of as much as 100 mC per liter had no demonstrable effect on growth or acid production over a period of 72 h [6]. Incubation was carried out in an air incubator for 17 h at 37°C. In order to increase the utilization of ^{32}P added to the medium inorganic phosphate was excluded from the salt mixture, since preliminary experiments had shown that the phosphorus present in Bactopeptone and liver furnished sufficient total phosphorus (0.003 %) for normal growth.

At the end of the cultivation the bacteria were separated from the medium in Sharples centrifuges. The usual yield of fresh bacteria from a single cultivation of 90 l was about 400—500 g. This material was washed two times with cold 10 % TCA. Lipids were extracted by treatment two times with a mixture of ethanol and ether in the ratio 3:1 at 40 to 50°C for 15 min and then with ether. Nucleic acids were extracted according to Schneider [7] for 15 min at 90°C with 5 % TCA. The residue was then treated with ethanol-ether (3:1) and ether and taken as the Schneider protein fraction. The normal yield was between 75—90 g.

After hydrolysis with 10 volumes of 2 N HCl per g dry protein for 20 h at 100°C insoluble humin was removed by centrifugation. The centrifugate was evaporated to dryness *in vacuo* three times with intermittent addition of water. The dissolved hydrolysate was usually mixed with the hydrolyzed

* 4 g of $MgSO_4 \cdot H_2O$, 1 g $FeSO_4 \cdot 7\ H_2O$, 1 g of NaCl, 0.1 g of $MnSO_4 \cdot H_2O$ were dissolved to 100 ml.

protein from an unlabeled batch of cells. Each half of the mixed hydrolysates was applied to a Dowex 50 column. The diameter was 6.5 and column length about 50 cm. Elution was carried out with 0.01 N HCl. A typical elution curve is given in a previous paper [1]. Irrespective of column dimensions MurP was obtained in the third column volume of eluate.

Excess of HCl was removed as described above and the residue, about 0.5 g by weight, was dissolved in a small volume of water and applied to a Dowex 1 formate (2 % DVB) column. (Dimensions 2 cm × 45 cm). Elution was carried out with a gradient 0→1 M formic acid and with a volume of 200 ml in the mixer. When the acidity in the eluate reached a value which corresponded to 0.60 M formic acid MurP appeared. For rechromatography a constant concentration of 0.5 M formic acid was sometimes used. With this system MurP appeared after 4.0 column volumes when it was chromatographed in a 1.0 cm × 43 cm column of Dowex 1. The usual yield of MurP from the two batches of cells was about 150 mg. For the following analysis about 1 g of MurP was prepared.

Dephosphorylation of MurP was at first performed in sealed glasstubes with 2 N HCl at 120°C and for 20 h. In this procedure a considerable part of Mur was destructed. It was found that MurP like O-phosphorylserine was hydrolyzed by prostate phosphatase. For that purpose an enzyme was used which had been purified according to Boman [8]. The digestion was performed in a 1.6 % solution of the substrate after pH was adjusted to 5.0 with trimethylamine. The reaction was followed by ionophoresis runs at pH 5 in a 0.1 M solution of pyridine-acetic acid. With the amounts of enzyme used it was found to be complete after 5 h of digestion at room temperature.

Crystalline phosphorylmuramic acid was obtained by slow evaporation of water solutions and from ethanol-water solutions as small needleshaped crystals. It was not possible to get crystals large enough for single crystal X-ray analysis. Under similar conditions Mur gave relatively large rods.

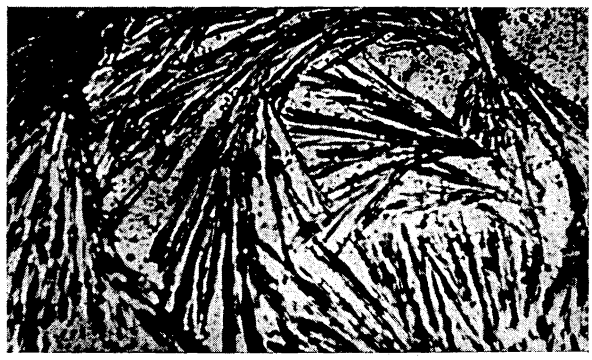

Fig. 1. A photograph of phosphorylmuramic acid isolated from the "protein fraction" of *L. casei*. Crystallized from a weak formic acid solution.

IDENTIFICATION AND CHARACTERIZATION

The elementary analysis first obtained showed that the compound must be a carbohydrate derivative containing nine carbon atoms. Sialic acid [9] and muramic acid are the most well known compounds with such a constitution. Different colour reactions described below were in favour of muramic acid. Against identity with sialic acid also was the high acid stability of our isolated compound. The following different methods were used for identification of the compound with muramic acid.

Elementary analysis

The data obtained are given in Table 2. The determinations of C, H, O, N were made by Mr. W. Kirsten at this institute and by Dr. A. Bernhardt, Mikroanalytisches Laboratorium im Max-Planck-Institut für Kohlenforschung, Mühlheim (Ruhr), Western Germany. Phosphorus was analysed according to a modification of Martin and Doty [10].

Table 2. Elementary analyses of the phosphorylated and dephosphorylated compound.

MurP:									
$C_9H_{18}O_{10}NP \cdot H_2O$ (349)	Calc.	C	30.95	H	5.77	O 50.40	N	4.01	P 8.87
	Found	1.						4.02	
								4.01	
		2.	30.55		5.94			3.94	
		3.							9.43
Mur:									
$C_9H_{17}O_7N$ (251)	Calc.	C	43.02	H	6.82	O 44.60	N	5.58	
	Found	1.	42.43		6.61	44.28		5.82	
		2.	41.44		7.09	—		5.76	
		3.	41.86		6.74	—		5.55	

Paper ionophoresis and paper chromatography

For ionophoresis Whatman paper No. 3 was used and for chromatography mostly No. 1. The ionophoretic migration of MurP in comparison to SerP is shown in Table 3. The dephosphorylated compound showed the same mobility as glycine by ionophoresis at pH 5. The solvents for the paper chromatographic identification of this compound are listed in the preceding paper [1]. The second solvent mentioned there was not used. Fig. 2 is a photograph of

Table 3. The ionophoretic mobility of MurP at pH 3 and 5 compared with SerP. + and − indicate that the substances move toward the anode and the cathode, respectively. Figures are given in cm.

	MurP	SerP
Sodium citrate − citric acid, pH 3	−4.0	+1.8
Pyridine acetate − acetic acid, pH 5	+14.0	+18.3

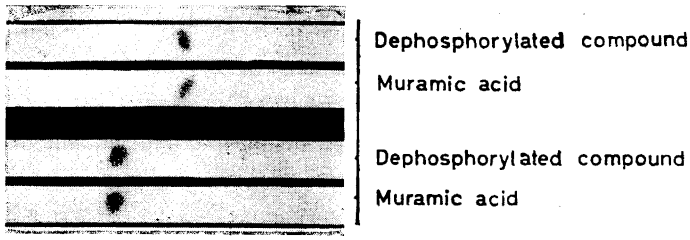

Fig. 2. Two one-dimensional chromatograms run with *iso*-butyric acid-NH_3-water (below) and *n*-butanol-acetic acid-water (above) showing that the dephosphorylated compound gives a spot identical with muramic acid.

a chromatogram with the dephosphorylated compound and Mur * running parallely. In the four solvents identical R_F values for the two compounds were obtained.

X-Ray diffraction studies

X-Ray powder diagram of MurP was obtained with a Debye-Scherrer camera but for the dephosphorylated compound a Guinier camera was used. Single crystal studies of Mur was done at the Department of Inorganic Chemistry, University of Uppsala **. The diagram of Mur supplied by Dr. Dark was identical with that obtained by enzymatic dephosphorylation of the phosphorus compound from the rest protein fraction (Fig. 3).

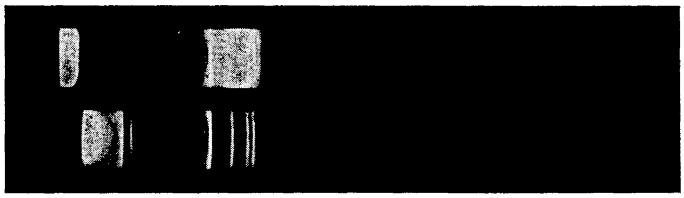

Fig. 3. X-Ray powder diagram of the dephosphorylated compound (below) is identical with that of muramic acid (above). (Some impurity in muramic acid has given a few additional weak bands.)

The density of the small crystals obtained was determined with the flotation method. Table 4 gives the spacings obtained for the phosphorylated and dephorsphorylated substances. The crystals of Mur belonged to the orthorhombic system with the cell dimensions: $a = 5.406$ Å; $b = 10.40$ Å, $c = 20.48$ Å. The density was 1.41. Calculated for four molecules per unit cell the formula weight will be 244.5 (calc. for $C_9H_{17}O_7N$ 251.24).

* The authors want to express their gratitude to Dr. F. A. Dark, the Microbiological Research Establishment, Porton, England, for the gift of a sample of muramic acid.

** The authors want to express their thanks to Dr. Abrahamsson who carried out this study.

Table 4. X-Ray diffraction data obtained from the phosphorylated and dephosphorylated compound.

Phosphorylmuramic acid		Muramic acid			
observed spacings		observed spacings		calculated spacings	
intensity	Å	intensity	Å	Å	index
v.w.	3.20	s	10.21	10.21	002
w	3.38	s	9.35	9.28	011
w	3.53	s	5.24	5.20	020
s	4.11	s	4.69	4.67	111
s	4.68	s	4.35	4.34	112
w	5.43	s	4.24	4.24	103
m	6.03	v.w.	4.19	—	—
w	6.73	m	3.93	3.92	113
v.w.	10.43	s	3.72	3.72	104
m	12.22	m	3.51	3.50	114
		w	3.46	—	—
		w	3.41	3.41	006
		v.w.	3.32	—	—
		v.w.	3.24	3.24	016
		m	3.04	3.02	124
		v.w.	2.89	2.89	131
				2.89	106
		w	2.85	2.85	026
		w	2.71	2.70	200
		v.w.	2.78	2.78	116
		w	2.67	—	—
		w	2.66	—	—
		w	2.56	2.56	008
		w	2.45	—	—
		v.w.	2.41	—	—
		v.w.	2.39	2.39	204
		v.w.	2.34	2.34	222

s = strong, m = medium, w = weak, v.w. very weak

Electrometric titration

The dissociation constants of MurP were determined by titration with 1.0 N sodium hydroxide added with an Agla micro burette. pH was measured with a direct reading pH meter (Electronic Instruments Ltd, Richmond, England Model 23 A). 38.05 mg MurP was titrated. The pK_s-values for the different steps can be seen from the following formula (Fig. 4). The corresponding values for L-O-phosphorylserine and L-O-phosphorylthreonine were 2.1; 5.7; 9.7; and 2.8; 6.1; 10.1. The dissociation constants of DL-phosphorylserine were determined by Österberg [11] with somewhat divergent values. The titration gives a molecular weight of 355 for MurP.

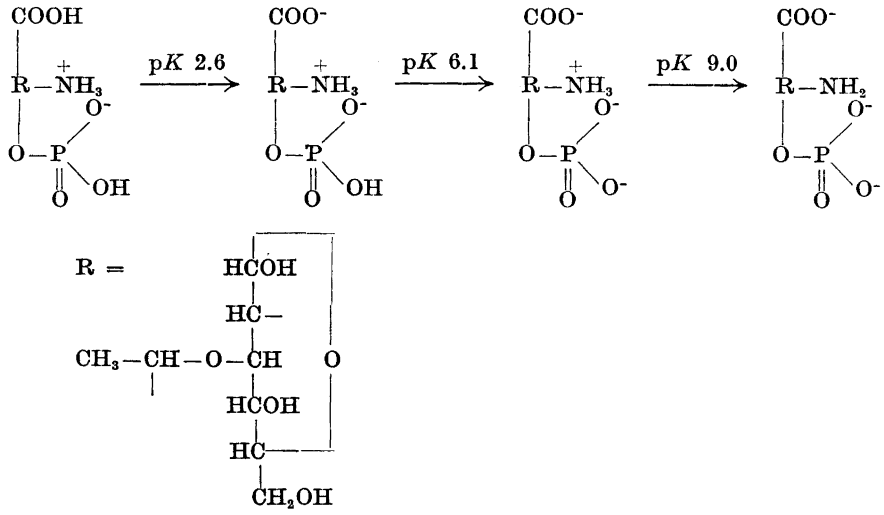

Fig. 4. The different steps of the protolysis of phosphorylated muramic acid.

Optical rotation

For MurP the following value was obtained: $\alpha_D^{25} + 3.43°$ (water; *l*,1; *c*, 4.21) which corresponds to

$$[\alpha]_D^{25} + 81.5°; [M]_D^{25} + 285°$$

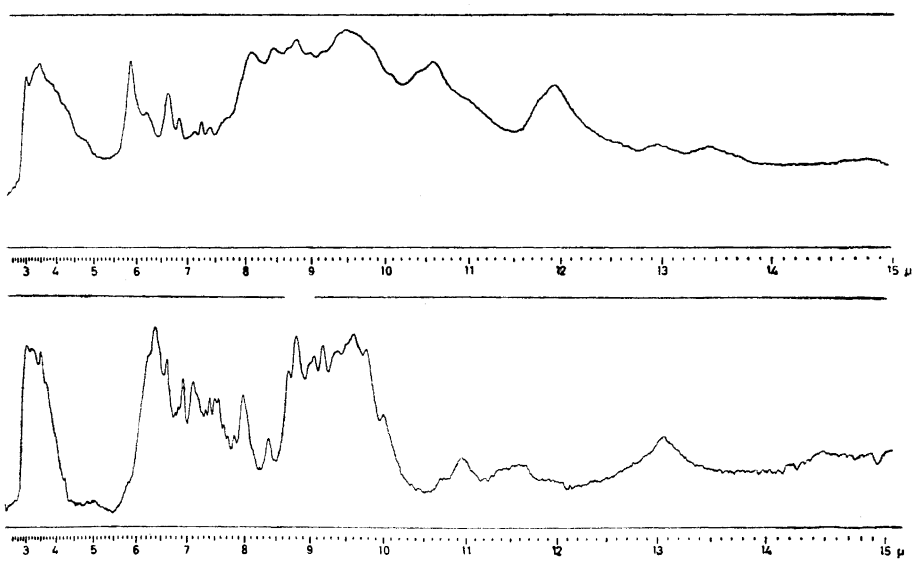

Fig. 5. Infrared absorption curves for MurP (above) and Mur.

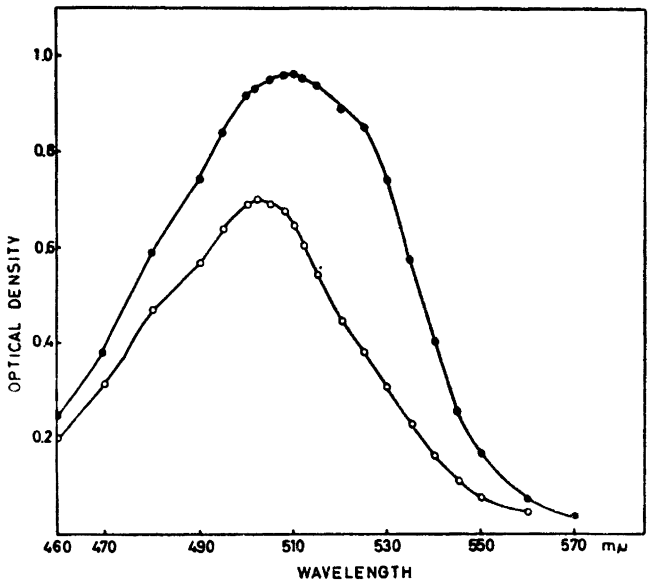

Fig. 6. Absorption curves of the colours obtained from Mur and MurP with the Elson-Morgan reaction. For -●-●- 1.94 μmole of MurP, for -O-O- 2.8 μmoles of Mur.

Infrared absorption analyses of the dephosphorylated compound and Mur were performed and showed identity. The absorption curves of Mur and MurP are shown in Fig. 5 *.

Colour reactions

The Elson-Morgan reaction was performed according to Blix' modification [12]. Absorption curves were drawn for both Mur and MurP after determinations with a Beckman DU spectrophotometer (Fig. 6). The displacement of the absorption maximum below 510 mμ is characteristic for a 3-substituted hexosamine [13]. On paper chromatograms MurP gave a positive reaction with ammoniacal $AgNO_3$ and also with anilinhydrogenphthalate a positive reaction was obtained [14]. Likewise on paper chromatograms both MurP and Mur were detected in very small amounts (2 and 1 μg, respectively) with ninhydrin reagent, 0.4 % solution in *iso*propanol-water (10:90).

DISCUSSION

To our knowledge protein-bound or free MurP has not been described. The exact position of the phosphorus linkage in MurP has not been certainly proved but according to all observations described in the previous sections

* The authors are indebted to Dr. Ingrid Fischmeister at this Institute for these analyses.

(acid stability and ninhydrin reaction) the phosphorus ought to be bound to the sixth carbon atom of the glucosamine moiety. The half life times of glucose-1-phosphate and glucosamine-1-phosphate in 1 N HCl at 100°C are about 1 and 4 min, respectively. For glucose-6-phosphate the corresponding figure is 1 300 min [15].

Mur is a major constituent of the bacterial cell wall [16] and according to theories put forward by Park and Strominger [17], uridine diphosphate-Mur and uridine diphosphate-Mur-peptides accumulate in consequences of the interference by penicillin with the biosynthesis of the cell wall. An alternative hypothesis has been put forward by Trucco and Pardee[18]. It was suggested that penicillin causes formation of an enzyme that attacks the cell membrane rather than the cell wall and that disruption of the membrane results in the escape of the cell content.

The observations of Park and Strominger were carried out with *Staphylococcus aureus* and in the microorganism an uridine diphosphate-Mur-peptide had the same amino acid composition as the cell wall. In *L. casei* the amino acid pattern is more complicated [16]. Possibly in line with this observation we have found a series of uridine diphosphate-Mur-peptides from *L. casei* inhibited by penicillin. These will be described in a following paper where the isolation of some Mur-P-peptides from the protein residue also will be given.

Regarding the role of MurP phosphorus in the phosphoproteins of *L. casei* it is possible that it has the structural function to cross-link peptide chains. Mur may also represent the active site in transphosphorylating enzymes [19] located either to cell plasma or cell walls. The relatively low turnover reported earlier makes it probable that most of the phosphorus atoms have a structural function. The mechanism for the synthesis of protein bound MurP has not been investigated more closely. It has so far not been possible to isolate free MurP. It cannot be excluded that uridine diphosphate-MurP may be an intermediate in the biosynthesis of protein-bound MurP. In this connection it must be mentioned that Strominger [20] recently isolated uridine diphosphate acetylglucosamine phosphate from oviduct of laying hen. He also suggested that uridine nucleotides of similar types may be activated intermediates in the biosynthesis of glycoproteins and mucopolysaccharides. A preliminary note has been published that galactosamine phosphate should be present in the hydrolysate of bone tissue [21]. More recently hexosamine phosphate has also been isolated in the hydrolysate of protein residue form *E. coli* [22].

REFERENCES

1. Ågren, G., de Verdier, D.-H. and Glomset, J. *Acta Soc. Med. Upsaliensis* **60** (1955) 30.
2. Ågren, G., de Verdier, C.-H. and Glomset, J. *Rev. Fermentations et inds. aliment.* **11** (1956) 234.
3. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 1570.
4. Kent, L. H. *Biochem. J.* **67** (1957) 5P.
5. Ågren, G. *Acta Physiol. Scand.* **17** (1949) 55.
6. Ågren, G., Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **9** (1955) 196.
7. Schneider, W. C. *J. Biol. Chem.* **161** (1945) 293.
8. Boman, H. G. *Biochim. et Biophys. Acta* **16** (1955) 295.
9. Blix, G., Lindberg, E., Odin, L. and Werner, I. *Acta Soc. Med. Upsaliensis* **61** (1956) 1.

10. Martin, J. B. and Doty, D. M. *Anal. Chem.* **21** (1953) 965.
11. Osterberg, R. *Nature* **179** (1957) 476.
12. Blix, G. *Acta Chem. Scand.* **2** (1948) 467.
13. Cifonelle, J. A. and Dorfman, A. *J. Biol. Chem.* **228** (1957) 547.
14. Cramer F. *Papierchromatographie*, Verlag Chemie, Weinheim 1953.
15. Leloir, L. F. and Cardini, C. E. in Colowick S. P. and Kaplan, N. O. *Methods of Enzymology*. Acad. Press, New York 1955, Vol. I p. 51,
16. Cummins, C. S. and Harris, H. *J. Gen. Microbiol.* **14** (1956) 583.
17. Park, J. T. and Strominger, J. L. *Science* **125** (1957) 99.
18. Trucco, R. E. and Pardee, A. B. *J. Biol. Chem.* **230** (1958) 434.
19. Ågren, G. *Acta Univ. Upsaliensis* **1958**, No. 5.
20. Strominger, J. L. *Biochim. et Biophys. Acta* **17** (1955) 283.
21. DiStefano, V., Neuman, W. F. and Rouser, G. *Arch. Biochem. Biophys.* **47** (1953 218.
22. Ågren, G. *Acta Chem. Scand.* **12** (1958) 1352.

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