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183. Tadashi Okabayashi and Eitaro Masuo : Occurrence of Nucleotides in the Culture Fluid of Microörganisms. II.*1 The Nucleotides in the Broth of *Brevibacterium liquefaciens novo sp.**2

(*Research Laboratory, Shionogi & Co., Ltd.**3)

Previous investigation with nonexacting purine auxotrophs of *Escherichia coli* revealed that a considerably large number of bacterial strains excrete purine and pyrimidine compounds into their culture fluids.*1 Examination of ultraviolet-absorbing substances in the broth of some bacteria by chromatography on paper suggested that the substances were mainly nucleotides having adenine, guanine, uracil, or cytosine as a base component. In the present work a more detailed study on the nucleotides in the broth of microörganism was undertaken using *Brevibacterium liquefaciens novo sp.*

A nucleotide mixture obtained from the culture fluid of *Brevibacterium liquefaciens novo sp.* by adsorption with charcoal, followed by elution with 50% ethanol containing 2% of conc. ammonia and concentrated *in vacuo*, was run through an anion-exchange resin (Dowex 1-X2, chloride form) and eluted with solutions of decreasing pH and increasing chloride concentration. The fractions corresponding to each peak were neutralized with calcium hydroxide gel to pH 7.0, evaporated to dryness, and the product was isolated as a calcium salt. In the case where further purification was desired, the peaks were submitted to rechromatography with a mixture of hydrochloric acid and sodium chloride. The nucleotides were adsorbed on a small column of charcoal, eluted with ethanol-ammonia, and were isolated as lyophilized ammonium salt. Each nucleotide thus obtained was analyzed so that the substance corresponding to each peak could be identified.

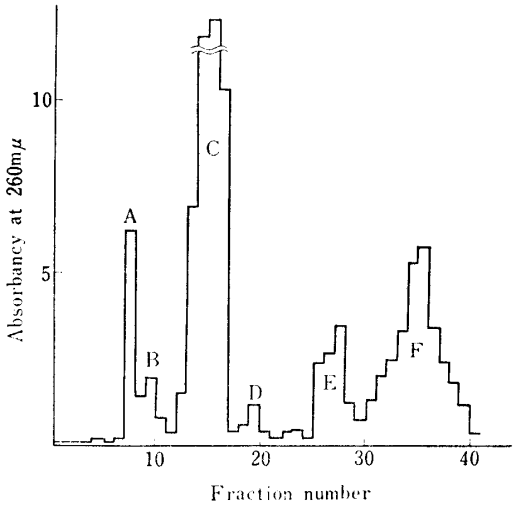

Fig. 1.

Gradient Elution of Nucleotides in the Culture Fluid of *Brevibacterium liquefaciens novo sp.*

Five hundred cc. of broth of shaken culture of *Brevibacterium liquefaciens novo sp.* was treated as described in the text and eluted from Dowex 1-X2 (chloride, 200~400 mesh). Column, 2.8×35 cm.; fractions, 30 cc.; mixing chamber, 1.12 L. filled with 0.005*N* HCl; reservior with 0.01*N* HCl +0.1*N* $CaCl_2$.

Fig. 1 which gives the most typical elution pattern shows that a variety of nucleotides occur in the culture fluid of *Brevibacterium liquefaciens novo sp.* This result is consistent with the result obtained by paper chromatography given in the previous paper.*1 Nucleotides identified in this experiment were 5'-CMP,*4 5'-AMP (peak C, 400 μ*M* in 1 L. of

*1 Part I : This Bulletin, 8, 1084(1960).
*2 A preliminary account of this work was published in this Bulletin, 8, 370(1960).
*3 192 Imafuku, Amagasaki, Hyôgo-ken (岡林 直, 増尾栄太郎).
*4 Abbreviations : 5'-AMP, adenosine-5'-phosphate; 5'-CMP, cytidine-5'-phosphate; 5'-UMP; uridine-5'-phosphate; ADP, adenosine-5'-pyrophosphate; CDP, cytidine-5'-pyrophosphate; UDP, uridine-5'-pyrophosphate.

broth), 5′-CDP (peak E, 60~90 μ*M*), 5′-UMP (peak F_1, 100 μ*M*), 5′-ADP (peak F_2a, 25 μ*M*) and a UDP-acetylhexosamine-peptide compound (peak F_2b, UDPX, 15 μ*M*). The structure of UDPX has not been clearly elucidated. It contains a peptide consisting of three amino acids, alanine, lysine, and glutamic acid. It also contains a substance positive to Elson-Morgan reaction.

As previously described,*[1] excretion of nucleotides from the cells of microörganisms under various circumstances has been reported by several investigators. Among these studies a phenomenon observed by Higuchi, *et al.*[1,2] resembles the present observation in some respects. They reported that a considerable amount of nucleotides were excreted after washed yeast cells were suspended in buffer solutions containing chelating agents. From the fact that various 2′- or 3′-mononucleotides, oligonucleotides, and polynucletides were detected (5′-mononucleotides were not obtained with the exception of a trace of 5′-CMP) they deduced that the excretion of nucleotides is due to decomposition of RNA by activated RNase by the action of chelating agents.

Only a little effort has been made to elucidate the mechanism of the present experiment. It is pertinent to assume, however, that an alternative mechanism is possible in this phenomenon, this being indicated by the nucleotides excreted as nucleoside 5′-phosphates.

The isolation of UDPX is interesting not only because this seems to be the first isolation of a UDP-acetylhexosamine-peptide compound from the culture fluids of microörganisms but also because this seems to offer a clue for elucidation of the mechanism for excretion of nucleotides. Evidence obtained up to the present indicates that UDPX closely resembles one of the UDP-acetylmuramic acid compounds, which were shown by Park, Strominger[3~6] and other investigators[7] to be significant as intermediates of bacterial cell-wall materials. An attempt is therefore being made to identify UDPX and to elucidate its significance in the excretion of nucleotides in the broth of *Brevibacterium liquefaciens novo sp.*

Experimental

Materials and Methods

Bacterial Strain and the Medium—A bacterial strain, which was described in a previous paper as strain 123-3, was designated as *Brevibacterium liquefaciens novo sp.* Taxonomical study of this microörganism will be given elsewhere. A medium listed in Table I was used throughout this work.

Table I. Composition of the Medium (1 L.)

K_2HPO_4	3.5 g.	*p*-Aminobenzoic acid	1 γ
KH_2PO_4	1.5 g.	Pyridoxine-HCl	200 γ
Na citrate	0.5 g.	Nicotinic acid	20 γ
$MgSO_4$ aq.	0.1 g.	Biotin	2.5 γ
$(NH_4)_2SO_4$	1.0 g.	Thiamine-HCl	100 γ
Vitamin-free casamino acid	20 g.	Riboflavin	100 γ
Glucose (sterilized separately)	20 g.	Ca-pantothenate	20 γ

Paper Chromatography—In one-dimensional, descending paper chromatography, Toyō Roshi No. 51A filter paper and the following solvent systems were used.

(a) iso-PrOH-H_2O (7:3) -NH_3 (as vapor phase)[8]

1) M. Higuchi, T. Uemura : Nippon Nôgei-Kagaku Zasshi, **33**, 304, 821, 826(1959).
2) *Idem* : Nature, **184**, 1381(1959).
3) J. T. Park, J. L. Strominger : Science, **125**, 99(1957).
4) J. L. Strominger : J. Biol. Chem., **234**, 1520(1959).
5) J. L. Strominger, R. H. Threnn : Biochim. et Biophys. Acta, **36**, 83(1959).
6) J. L. Strominger, J. T. Park, R. E. Thompson : J. Biol. Chem., **234**, 3263(1959).
7) E. Ito, N. Ishimoto, M. Saito : Arch. Biochem. Biophys., **80**, 431(1959).
8) R. Markham, J. D. Smith : Biochem. J., **52**, 552(1952).

(b) 95% EtOH-*N* $AcONH_4$ (75:30) buffer (pH 3.8)[9]
(c) 95% EtOH-*N* $AcONH_4$ (75:30) buffer (pH 7.5)[9]
(d) Saturated $(NH_4)_2SO_4$-*N* AcONa-iso-PrOH (80:18:2)[9]
(e) 70% *tert*-BuOH-0.8*N* HCl[10]

Paper chromatograms were irradiated with ultraviolet ray to locate nucleotides. The rate of movement of each nucleotide was expressed as $R_{adenosine}$ value (relative to Rf value of adenosine).

The following solvent systems were also used in cases where it was desirable to detect amino acid and hexosamine: *n*-BuOH-AcOH-H_2O (4:1:2),[11] PhOH-H_2O (8:2, NH_3 as vapor phase),[11] *sec*-BuOH-HCOOH-H_2O (75:15:10),[11] C_6H_5OH-*m*-cresol-borate buffer (pH 9.3) (25:25:7),[12] and 80% pyridine.[5] The dried chromatograms were sprayed with Ninhydrin or the Elson-Morgan reagent to reveal the substances.

Analytical Procedure—Phosphorus was determined by the method of Allen,[12] pentose was estimated by the orcinol reaction,[13] and carbazole reaction was carried out as described by Dische, *et al.*[14] to discriminate the 3'- and 5'-nucleotide.

As the stability of glycosidic linkage of pyrimidine nucleotides in hydrolytic procedure made it impossible to carry out the carbazole reaction directly, these nucleotides were reduced with Na-amalgam prior to the addition of carbazole reagent. The method which was first proposed by Haavaldsen, *et al.*[15] as a convenient pretreatment for determination of pentose was also found to be useful as a means of pretreatment for the carbazole reaction in pyrimidine nucleotides. HIO_4 oxidation was undertaken according to the method of Whitfeld[16] and the oxidation products were compared on paper chromatogram with solvent (a) with those obtained from authentic samples.

Isolation of Nucleotides from the Culture Fluid of *Brevibacterium liquefaciens novo sp.*—*Brevibacterium liquefaciens*** was cultured with shaking in the medium listed in Table I at 28° for 48 hr. The broth was centrifuged (7000 r.p.m.) to sediment bacterial cells. To 500 cc. of the supernatant, 15 g. of washed charcoal was added. After vigorous stirring for 1 hr., followed by standing for 30 min., the suspension was filtered through a Buchner funnel with a filter aid. The charcoal was washed thoroughly with H_2O, 0.2*M* EDTA (pH 7) solution, and H_2O. Elution of nucleotides from the charcoal was carried out with H_2O-EtOH-NH_3 mixture (50 cc. of 99% EtOH, 48 cc. H_2O, and 2 cc. 30% NH_3) in a column (diameter, 10 cm.) with a bed of Hyflo Super-cel. The effluent was evaporated to dryness *in vacuo* at below 35°. The residue was dissolved in a small amount of H_2O and the clear supernatant (pH 7.5) was submitted to ion exchange chromatography.

Column Chromatography—The nucleotides in the supernatant was adsorbed on a column of Dowex 1-X2 (chloride form, 200~400 mesh), the column was washed with H_2O, and eluted with the solvent system of HCl-$CaCl_2$ by gradient elution. The effluent was collected in 30-cc. fractions at the rate of 2.8 cc./min. Absorbancy at 260 mμ was measured with a Hitachi spectrophotometer and the nucleotides corresponding to the peaks were collected, neutralized with $Ca(OH)_2$ gel, evaporated *in vacuo* at below 30°, and isolated as Ca salts as described by Pontis, *et al.*[17] In case where further purification was desirable, rechromatograpy was carried out with a mixture of HCl and NaCl. The nucleotides were adsorbed on charcoal, followed by elution with EtOH-NH_3, and were recovered as ammonium salts.

Identification of Peaks from Column (Fig. 1)—Peak A (Unknown substance): This substance was not analyzed, as it gave a different spectrum to those of nucleotides.

Peak B (5'-CMP+unknown substance): Two spots (B_1 and B_2) were obtained by paper chromatography with the solvent (a). One spot (B_1) was cut out, eluted with H_2O, and was found to give a spectrum similar to that of cytidine. B_1 was indistinguishable from authentic 5'-CMP on paper chromatography with the solvents (b) and (c). Carbazole test revealed that B_1 was 5'-phosphate. From these results B_1 was tentatively identified as 5'-CMP. Another spot B_2 was not identified.

Peak C (5'-AMP, Table II): This had an adenine spectrum. Analysis gave a ratio of adenine (calculated from absorbancy)-organic P-ribose of 1:0.93:1.10 in agreement with the expected value for adenosine monophosphate. Peak C nucleotide gave the same $R_{adenosine}$ value as that of authentic 5'-AMP with four different solvent systems and the results with the solvent (d) indicated that the nucleotide is not 3'-AMP but 5'-AMP. Carbazole reaction gave a blue coloration having two peaks

9) A. C. Plandini, L. F. Leloir: *Ibid.*, **51**, 426(1952).
10) J. D. Smith, R. Markham: *Ibid.*, **46**, 509(1950).
11) K. Iwai, M. Kimura: "Jikken Kagaku Kôza," 235(1958). Maruzen Co., Tokyo.
12) R. J. L. Allen: Biochem. J., **38**, 858(1940).
13) W. R. Fernell, H. K. King: Analyst, **78**, 80(1953).
14) Z. Dische, E. Landsberg: Biochim. et Biophys. Acta, **24**, 193(1957).
15) L. Haavaldsen, S. Laland, J. M. Mckee, E. Roth: *Ibid.*, **33**, 201(1959).
16) P. R. Whitfeld: Biochem. J., **58**, 390(1954).
17) H. G. Pontis, N. L. Blumson: Biochim. et Biophys. Acta, **27**, 618(1958).

Table II. Analytical Data of Peak C

Substance	Nucleoside[a]	Nucleoside/organic P ratio	$R_{adenosine}$ with the solvent[b] a	b	c	d	Absorption maximum in carbazole reaction (mμ)
Peak C	Adenosine	1 : 0.93	0.39	0.81	0.44	2.16	600, 710
5′-AMP			0.39	0.82	0.44	2.14	600, 710
3′-AMP			—	—	—	1.2	540

a) Deduced from ultraviolet absorption spectra in acid (0.1*N*), neutral, and alkaline solutions, and paper chromatography with the solvent (e) after the sample was hydrolyzed with 1*N* HCl at 100°.

b) $R_{adenosine}$ value of each nucleotide varied because of the lack of temperature control. In all cases authentic nucleotides were run as controls.

at 710 and 600 mμ, and this result indicates that peak C is 5′-AMP, not 3′-AMP. Following oxidation of peak C with $NaIO_4$, the oxidation products were compared with those of authentic 5′ and 3′-AMP. Fig. 2 shows that peak C is not 3′-AMP but 5′-AMP.

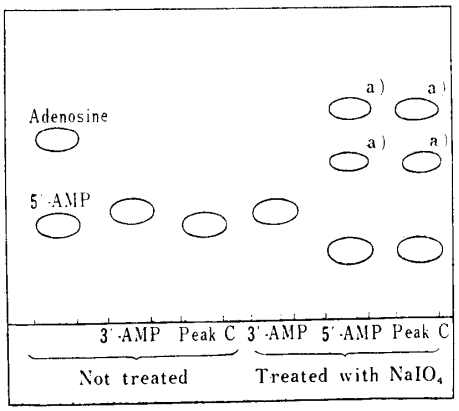

Fig. 2.
Periodate Oxidation of Peak C

a) Faint spots

Table III. Analytical Data of Peak E

Substance	Nucleoside[a]	Nucleoside/organic P/labile P ratio	$R_{adenosine}$ with the solvent[b] a	b	c	d	Absorption maximum in carbazole reaction (mμ)
Peak E_1	Cytidine	1:0.96	0.50	0.49	0.52	5.4	600, 710
Peak E_2	Cytidine	1:2.08:0.9	0.32	0.33	0.37	5.7	600, 710
Peak E_2[c] hydrolyzed	Cytidine		0.51	0.49	0.51	5.4	600, 710
CDP			0.32	0.33	0.38	5.7	600, 710
3′-CMP			0.56	0.55	0.54	5.1	540
5′-CMP			0.53	0.44	0.50	5.3	600, 710

a), *b*) Same as in Table II.

c) Hydrolyzed with 1*N* HCl at 100°C for 1 hr.

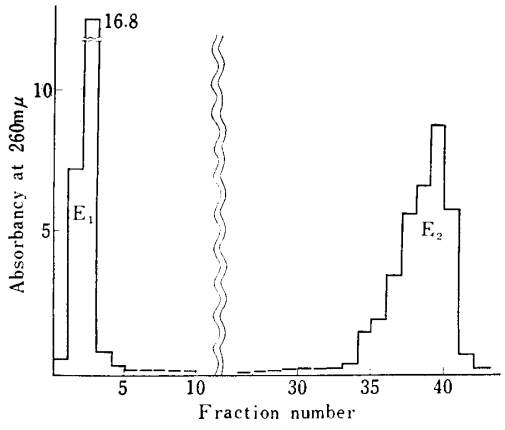

Fig. 3.
Rechromatogram of Peak E on Dowex-1

About 110 μ*M* of crude cytidine nucleotide was submitted to gradient elution. Column 1.0 × 40 cm.; fractions, 10 cc. each; mixing chamber filled with 0.01*N* HCl + 0.025*N* NaCl; reservior with 0.01*N* HCl + 0.1*N* NaCl.

Peak E (5′-CDP, Table III): Rechromatography of peak E (Fig. 3) gave two peaks (E_1 and E_2), both of which gave typical cytidine spectrum. Both peaks gave cytidylic acid on paper chromatography with the solvent (e), after they were hydrolyzed with 1*N* HCl for 1 hr. at 100°. Analysis for peak E_2, which eluted slower from the column, gave a ratio of cytidine-organic P-labile P of 1:2.08:0.9. The values agree with those of cytidine diphosphate. It moved at the same speed as 5′-CDP (Sigma) on paper chromatograms with the solvent system of (a), (b), (c), and (d). The faster eluted peak, E_1, yielded a ratio of cytidine-organic P of 1:0.96. Examination of E_1 on paper chromatography revealed that this is 5′-CMP. It is supposed, therefore, that peak E_1 is a hydrolyzed product of E_2 in the course of concentration and recovery of E_2.

E_1, E_2, and HCl-hydrolyzed product of E_2 were reduced with Na-amalgam and submitted to carbazole reaction and all were found to give a blue color (peaks 710 and 600 mμ). This result indicates that peak E_2 is a 5′-nucleotide.

Table IV. Analytical Data of Peak F

i) F_1 and F_2b

Substance	Nucleoside[a)]	Nucleoside/organic P/labile P ratio	$R_{adenosine}$ with the solvent[b)] a	b	c	d	Absorption maximum in carbazole reaction (mμ)
F_1	Uridine	1.0:1.0	0.58	0.94	0.61	4.4	600, 710
F_2b	Uridine	1:1.95:0.96	0.72	0.84	0.80		600, 710
F_2b[c)] hydrolyzed	Uridine		0.60	0.93	0.60		600, 710
3′-UMP			0.62	0.98	0.66		540
5′-UMP			0.59	0.93	0.59	4.4	600, 710

ii) F_2a

Substance	Nucleoside[a)]	Nucleoside/organic P/labile P ratio	$R_{adenosine}$ with the solvent[b)] a	b	c	Absorption maximum in carbazole reaction (mμ)
F_2a	Adenosine	1:2.05:0.97	0.49	0.62	0.28	600, 710
5′-AMP			0.55	0.77	0.41	
ADP			0.49	0.63	0.28	
ATP			0.18	0.35	0.18	

a), *b*) Same as in Table II.

c) Hydrolyzed with *N* HCl at 100° for 1 hr. Milder acid-hydrolysis gave UDP as major product and 5′-UMP as minor product, which were confirmed with solvent (c).

Peak F (5′-UMP+ADP+UDP-acetylhexosamine-peptide (UDPX) (Table IV): The nucleotides in peak F were poorly separated by the column chromatography employed. Examination by paper chromatography with the solvents (a) and (c) gave three nucleotides. Each spot was eluted, hydrolyzed with 1*N* HCl, and examined on paper chromatogram with the solvent (e). Two of these nucleotides were found to be uridine nucleotide, and the other adenine nucleotide. Combination of fractional precipitation in EtOH and rechromatography on Dowex-1 separated these nucleotides from each other. Ca salts of peak F nucleotides were dissolved in cold dil. HCl, the solution was neutralized with $Ca(OH)_2$ gel to pH 7.8~8.0, and a calibrated amount of EtOH was added. Almost all adenine nucleotide (F_2a) and most of uridine nucleotide (F_1) were precipitated in 0~60% EtOH fraction. These were separated by rechromatography on Dowex-1 with a mixture of HCl and NaCl (Fig. 4). The remainder in the supernatant contained most of the UDP-acetylhexosamine-peptide compound (F_2b) and a small part of F_1. These were also separated by rechromatography (Fig. 5).

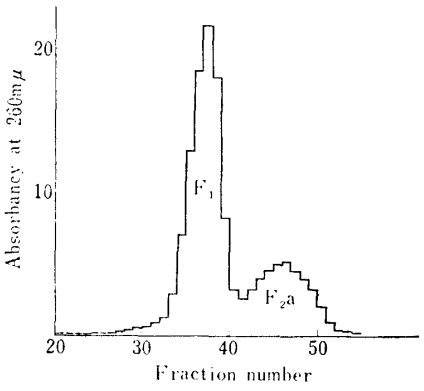

Fig. 4.

Rechromatography of 60% Ethanol-precipitated Fraction on Dowex-1

Column, 1.5×30 cm.; fractions, 10 cc. each; mixing chamber 1.12 L. filled with 0.035*N* NaCl+0.01*N* HCl; reservoir with 0.01*N* HCl+0.1*N* NaCl

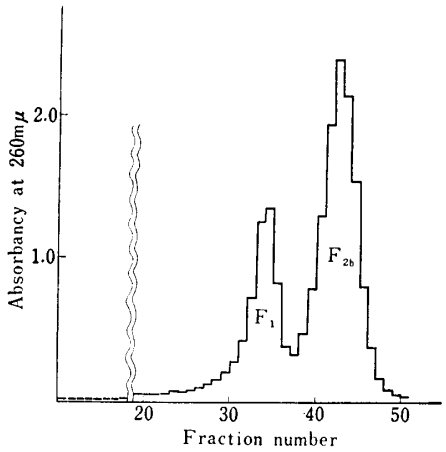

Fig. 5.
Rechromatography of the Supernatant

Exprimental conditions same as in Fig. 4.

The faster-eluted peak F_1 (Figs. 4 and 5), gave an analytical ratio of uridine-organic P of 1.0:1.0. From the $R_{adenosine}$ values on paper chromatograms and the color developed in carbazole reaction, this was identified as 5′-UMP (Table IV, (i)). HIO_4 oxidation also confirmed it.

The slower eluted peak F_2a (Fig. 4) gave an analytical ratio of adenosine-organic P-labile P of 1:2.05:0.97 in good agreement with ADP. It was inseparable with authentic ADP on paper chromatogram with the solvents (a), (b), and (c) (Table IV, ii). Carbazole test showed that this was 5′-phosphate.

F_2b (Fig. 5) gave a ratio of uridine-organic P-labile P of 1:1.95:0.96. On paper chromatogram, it migrated as one UV-absorbing spot, which gave a feeble purple color on spraying with Ninhydrin reagent. It migrated faster than 3′-UMP on paper chromatogram with alkaline and neutral solvents ((a) and (c)). On hydrolysis with *N* HCl at 100°, it gave 5′-UMP (see Table IV, i). On hydrolysis with 6*N* HCl at 100° for 15 hr., F_2b gave 4 Ninhydrin-positive spots, three of which were identified as lysine, alanine, and glutamic acid (see Rf values in Table V). The fourth spot of Rf 0.78 on paper chromatogram with 80% pyridine, also gave a salmon pink coloration on spraying with Elson-Morgan reagent.

Table V. Rf Value of Ninhydrin-positive Spots after UDPX was hydrolyzed with 6*N* HCl

	sec-BuOH-HCOOH-H_2O	C_6H_5OH-H_2O (8:2) NH_3 (vapour phase)	C_6H_5OH-*m*-cresol-borate buffer (pH 9.3)	BuOH-AcOH-H_2O	80% Pyridine
Alanine	0.45	0.53	0.42	0.35	0.35
Glutamic acid	0.34	0.25	0.07	0.32	0.235
Lysine	0.1	0.73	0.14	0.16	0.025
Hydrolyzed UDPX	0.43	0.56	0.41	0.36	0.34
	0.33	0.25	0.06	0.31	0.22
	0.11	0.71	0.14	0.19	0.025
					0.76

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Summary

Nucleotides in the culture fluid of *Brevibacterium liquefaciens novo sp.* were adsorbed on charcoal, eluted with 50% ethanol containing 2% of conc. ammonia, and were submitted to column chromatography on Dowex-1 (chloride). Each nucleotide was isolated as calcium or ammonium salt and identified. Following nucleotides were identified : Cytidine 5′-phosphate, adenosine 5′-phosphate, cytidine 5′-pyrophosphate, uridine 5′-phosphate, adenosine 5′-pyrophosphate, and a uridine 5′-pyrophosphate-acetylhexosamine-peptide compound. The last compound gave alanine, glutamic acid, lysine, and an Elson-Morgan reaction-positive substance on paper chromatogram after it was hydrolyzed by acid.

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