ANALOGS OF CARBOHYDRATE METABOLISM COENZYMES
VII. Synthesis of Isocytidine Diphosphate Glucose

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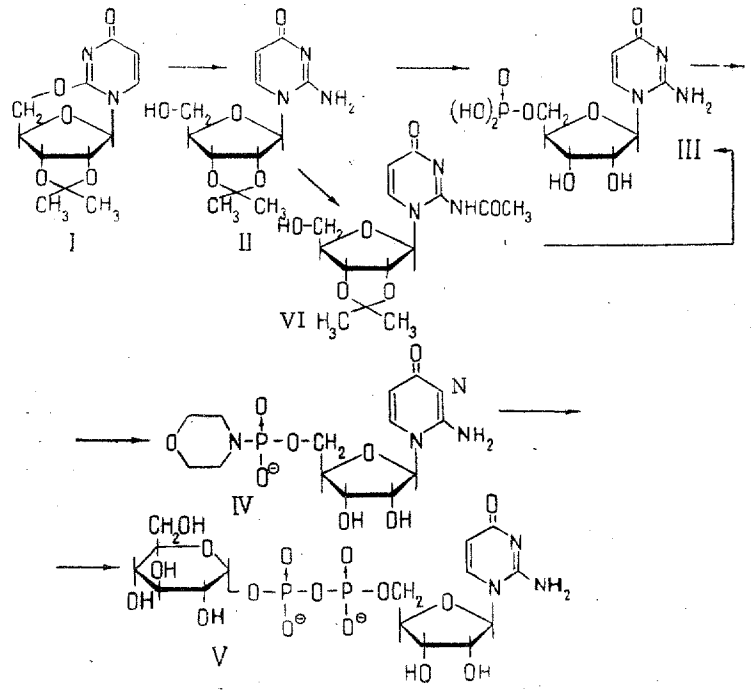
We have previously [1, 2] pointed out the importance of the imide grouping $O{=}C_2{-}N_3H{-}C_4{=}O$ of the uracil nucleus, which is capable of forming hydrogen bonds, in the biological activity of uridine diphosphate glucose (UDPG). According to a hypothesis in [2], the presence of this grouping is essential for the formation of the secondary structure of a nucleoside diphosphate sugar, determining the specificity of its biochemical properties. In preceding papers of this series the synthesis of analogs of UDPG modified at N_3 and C_4 of the uracil nucleus has been reported: 3-N-methyluridine diphosphate glucose [1], cytidine diphosphate glucose [3], and 4-thiouridine diphosphate glucose [4]. The study of their biochemical and chemical properties has enabled the following conclusions to be drawn:

1) Analogs of UDPG containing the grouping $N_3H{-}C_4{=}X$ (where X = O, S) in the uracil nucleus, which are capable of forming hydrogen bonds, can replace UDPG in four essentially different enzymatic reactions; a change in this grouping involving a loss of the capacity for forming hydrogen bonds destroys the biological activity of the compound [5, 6];

2) The catalytic hydrogenation of UDPG and its analogs with a nonmodified $N_3H{-}C_4{=}O$ group in the uracil nucleus takes place considerably more slowly than the hydrogenation of the corresponding nucleoside-5'-phosphates. This difference is not observed for analogs which are not capable of forming hydrogen bonds and are inactive in enzymatic reactions [7].

These conclusions agree completely with the hypotheses previously put forward on the existence of a secondary structure in nucleoside diphosphate sugars [2]. However, the answer to the question of the importance of a carbonyl group at C_2 of the uracil nucleus in the formation of the secondary structure and in the biological activity of UDPG requires the investigation of analogs of UDPG modified at C_2 of the pyrimidine nucleus. The present paper describes the synthesis of an analog containing a NH_2 group in the place of the carbonyl group at C_2 of isocytidine diphosphate glucose. Brief information on its synthesis has been published in [8].

The starting material for the preparation of isocytidine derivatives was 2', 3'-O-isopropylidene-O^2, 5'-cyclouridine (I). The synthesis of this compound has been reported by Brown, Todd, and Varadarajan [9], who obtained it by the action of silver acetate on 2', 3'-O-isopropylidine-5'-deoxy-5'-iodouridine in absolute methanol. On repeating this procedure, we found that in addition to compound (I) a considerable amount of a by-product having a lower polarity was formed. It is probable that this product, which we did not investigate in detail, arises through the opening of the ring of (I) under the action of methanol. In actual fact, when the reaction was carried out in acetonitrile the formation of the by-product was completely eliminated and the yield of (I) rose somewhat. Isocytidine diphosphate glucose was synthesized from substance (I) by the following route:



The conversion of compound (I) into 2', 3'-O-isopropylideneisocytidine (II) was carried out by treating its solution with ammonia in methanol by the procedure described by Brown and co-authors [9]. The absorption maxima of the UV spectrum of the reaction product showed characteristic changes when the pHs varied and were close to those given in the literature.

The phosphorylation of substance (II) encountered considerable difficulties. The use of Tener's method – phosphorylation with 2-cyanoethyl phosphate in the presence of dicyclohexyl carbodiimide [10] – did not give satisfactory yields of isocytidine 5-phosphate. The reaction of (II) with 2-cyanoethyl phosphate and dicyclohexylcarbodiimide under standard conditions (4 hours, 60°C) [4], followed by hydrolysis to eliminate the isopropylidene group by the action of concentrated formic acid in the cold (under standard conditions of hydrolysis the N-glycoside bond of the isocytidine ruptures completely) and treatment with a dilute solution of ammonia to eliminate the cyanoethyl group led to the formation of isocytidine-5'-phosphate (III) with a yield of 15%. Reducing the reaction time to 1.5 hr increased the yield to 25%. When N-acetylisopropylideneisocytidine (VI) was phosphorylated by means of 2-cyanoethyl phosphate and dicyclohexylcarbodiimide it was possible to isolate substance (III) with a yield of 40%. The product was formed as a result of the action of acetic anhydride and triethylamine on (II) in admixture with dioxane and dimethylformamide (cf. [12]). The behavior of the substance on paper and a study of its UV spectrum with change in pH agree with the structure assigned to compound (VI).

The best method of obtaining (III) proved to be the direct phosphorylation of isopropylideneisocytidine with pyrophosphoryl chloride. This reagent has been used previously with success for the synthesis of natural nucleoside-5'-phosphates [12]. The reaction of (II) with pyrophosphoryl chloride took 16 hr at room temperature; no side reactions were found. The 2', 3'-O-isopropylideneisocytidine-5'-phosphorodichloroate formed as an intermediate was hydrolyzed with water at −30°. The isopropylidene group was split off by keeping a strongly acid solution at room temperature for a short time; the N-glycoside bond did not hydrolyze during this treatment. The isocytidine-5'-phosphate was separated from isocytidine and phosphoric acid by ion-exchange chromatography on "Dowex-1" anion-exchanger (Cl^- form) and was isolated in the form of the lithium salt in 50% yield. From its chromatographic, electrophoretic, and spectroscopic properties the substance obtained corresponded to isocytidine-5'-phosphate.

Thus, we have succeeded in developing a method which can be used not only for the synthesis of analogs of UDPG but also in investigations on the relationship of structure and function in natural nucleosides.

The conversion of isocytidine-5'-phosphate into isocytidine-5'-phosphoromorpholidate (IV) was effected by the method of Khorana and coworkers, [13]; a sample of (IV) containing traces of (III) was used without further purification for reaction with α-D-glucose-1-phosphate by a known general method [14] with modifications [1, 3]. The final product of the whole synthesis, isocytidine diphosphate glucose (V), was purified by ion-exchange chromatography on DEAE-cellulose, and was then isolated as the lithium salt. For analysis and for biochemical investigation, compound (V) was converted into the sodium salt. The sample of (V) was homogeneous on chromatography and paper electrophoresis; its structure was shown on the basis of the criteria customary for such compounds: the UV spectrum, which agrees with the UV spectrum of (III), the electrophoretic mobility characteristic for a di-substituted pyrophosphate, and the nucleoside-glucose ratio after acid hydrolysis (found: 1:0.90). The yield of product (V) from (III) was 50%.

Preliminary results of the biochemical investigation of substance (V) showed that it is incapable of replacing UDPG in reaction with saccharososynthetase and UDPG-4-epimerase. This fact shows the improtance of the carbonyl group at C_2 in the biological activity of UDGP and agrees with the hypothesis previously expressed on the secondary structure of nucleoside diphosphate sugars [2].

Experimental

The systems used for paper chromatography were: 1) butan-1-ol—water (86:14); 2) ethanol – 0.5 M ammonium acetate solution, pH 7.5 (5:2). Buffer solutions: pH 7.5, 0.02 M solution of triethylammonium carbonate; pH 4.0, 0.0075 M triethylammonium acetate solution.

2', 3'-O-Isopropylidene-O^2, 5'-cyclouridine (I). a. A mixture of 2.36 g (6.1 mmole) of 5'-deoxy-5'-iodo-2', 3'-O-isopropylideneuridine [15], 4.44 g (26.5 mmole) of silver acetate and 500 ml of absolute methanol was boiled for 15 minutes without the access of moisture and filtered through Celite. Hydrogen sulfide was passed through the filtrate, the precipitate of silver sulfide was separated by filtration through Celite, and the solution was evaporated to dryness. The residue was recrystallized from alcohol. This gave 0.95 g (58%) of 2', 3'-O-isopropylidene-O^2, 5'-cyclouridine. The product was homogeneous on paper chromatography with R_f 0.53 (system 1). UV spectrum (in methanol): λ_{max} 237 mμ, λ_{min} 212 mμ, which agrees with literature data [9]. According to paper chromatography in system 1, the mother liquor contained a substance with R_f 0.85, in addition to isopropylidenecylouridine.

b. A mixture of 1.10 g (2.72 mmole) of 5'-deoxy-5'-iodo-2', 3'-O-isopropylideneuridine and 110 ml of dry acetonitrile was treated with 1.58 g (9.5 mmole) of silver acetate and was stirred for a night at room temperature without the access of moisture and light. The mixture was filtered through Celite and the residue was washed with 10 ml of

acetonitrile. The filtrate was evaporated to 10 ml and hydrogen sulfide was passed into it. The precipitate of silver sulfide was separated off by filtration through Celite and was washed with 20 ml of acetonitrile. The filtrate was treated with 50 ml of dry benzene and 100 ml of cyclohexane, and the mixture was allowed to stand overnight in a refrigerator. A precipitate of 0.393 g (53%) of 2', 3'-O-isopropylidene-O^2, 5'-cyclouridine was filtered off; when the mother liquor was evaporated a further 0.125 g of chromatographically homogeneous product was obtained. The total yield was 0.518 g (69%). From its chromatographic behavior and spectroscopic properties, the product was identical with that obtained by method a.

2', 3'-O-Isopropylideneisocytidine (II). A solution of 185.4 mg (0.69 mmole) of 2', 3'-O-isopropylidene-O^2, 5'-cyclouridine in 6.5 ml of absolute methanol was treated with 24 ml of a saturated solution of ammonia in absolute methanol and the mixture was left for 3 days at room temperature. Chromatography on paper in system 1 showed the rapid conversion of the 2', 3'-O-isopropylidene-O^2, 5'-cyclouridine (R_f 0.53) into 2', 3'-O-isopropylidene-2-O-methyluridine (R_f 0.79), which then gradually changed into 2', 3'-O-isopropylideneisocytidine (R_f 0.47). The reaction mixture was evaporated to dryness, and the residue was evaporated in vacuum to give a glass-like residue of 2', 3'-O-isopropylideneisocytidine. Yield 185.9 mg (96%). UV spectrum: in 0.1 N HCl, λ_{max} 257, 220 mμ, ε_{max} 7120, 9250; λ_{min} 239 mμ, ε_{min} 4940; in 0.1 N NaOH, λ_{max} 224 mμ, ε_{max} 15 300. Reference [9]: UV spectrum: in 0.1 N HCl, λ_{max} 256, 220 mμ, ε_{max} 7110, 8390, λ_{min} 239 mμ, 4790; in 0.1 N NaOH, λ_{max} 224 mμ, ε_{max} 16 500.

N-Acetyl-2', 3'-O-isopropylideneisocytidine (VI). A solution of 26.0 mg (0.094 mmole) of 2', 3'-O-isopropylideneisocytidine in 1.5 ml of dry dimethylformamide was treated with 1 ml of a 0.1 M solution of triethylamine in dry dioxane, and the mixture was left for 24 hr at room temperature. The reaction mixture was evaporated to dryness and was dried in vacuum over phosphorus pentoxide (60°, 0.1 mm) to eliminate the triethylammonium acetate. This gave 29.0 mg (96%) of chromatographically pure N-acetyl-2', 3'-O-isopropylideneisocytidine, mp 145-148°; R_f 0.88 (system 1). UV spectrum: in 0.1 N HCl and water, λ_{max} 257 mμ, ε_{max} 11 300, λ_{min} 234 mμ, ε_{min} 4900; in 0.1 N NaOH, λ_{max} 231 mμ, ε_{max} 12 200.

Isocytidine-5'-phosphate (III). a. Phosphorylation wtih pyrophosphoryl chloride. 416.7 mg (1.47 mmole) of ground 2', 3'-O-isopropylideneisocytidine cooled to −30° was treated with 920 mg (3.68 mmole) of pyrophosphoryl chloride [16]. The reaction mixture was allowed to warm up to room temperature with vigorous stirring and was left overnight at room temperature. The mixture was cooled to −30°, and 0.5 ml of water was added; it was then warmed to room temperature with vigorous stirring, diluted with 20 ml of water, and kept for 20 min at room temperature, after which it was neutralized with lithium hydroxide to pH 8.5. The small amount of solid material was filtered off, and the solution was diluted with water to 500 ml and passed through a column (19 × 3 cm) of "Dowex" 1 × 4 (200 × 400 mesh, Cl form). The column was washed with 300 ml of water (the isocytidine being eluted, $TOD_{246}1260$ = 0.22 mmole) and then with 0.003 N HCl solution. After the passage of 3200 ml of solution, the elution of isocytidine-5'-phosphate began, and was complete in the subsequent 1200 ml of solution. The fractions containing the isocytidine-5'-phosphate ($TOD_{246}4500$ = = 0.78 mmole; 53%) were combined, neutralized with lithium hydroxide, and evaporated to dryness. The residue was dried by two azeotropic distillations with a mixture of benzene and alcohol and dissolved in 5 ml of methanol, and the lithium salt of isocytidine-5'-phosphate was precipitated by the addition of 35 ml of acetone and 5 ml of ether. The precipitate was separated off by centrifuging and was reprecipitated twice more. The lithium salt of isocytidine-5'-phosphate was obtained, yield 240 mg (50%). The product was homogeneous on chromatography and paper electrophoresis; R_f 0.17 (system 2), R_{UMP} 1.0 (pH 7.5), R_{UMP} 0.65 (pH 4.0). UV spectrum: in 0.1 N HCl − λ_{max} 220, 256 mμ, ε_{max} 9 400, 7 400; λ_{min} 239 mμ, ε_{min} 5000; in water, λ_{infl} 255 mμ, ε_{infl} 5800; in 0.1 KOH, λ_{max} 226 mμ, ε_{max} 16 000; isosbestic point, 246 mμ, ε 5800 (value of the molar extinction calculated for $C_9H_{12}N_3O_8P{=}Li_2$, which corresponds to mol. wt. 335.0).

b. Phosphorylation with 2-cyanoethyl-phosphate and dicyclohexylcarbodiimide. A mixture of 66.2 mg (0.23 mmole) of 2', 3'-O-isopropylidineisocytidine and 0.92 ml of 1 M 2-cyanoethyl phosphate [10] solution in aqueous pyridine was dried by evaporating off dry pyridine three times and was dissolved in 10 ml of dry pyridine; 585 mg (2.75 mmole) of dicyclohexylcarbodiimide was added and the mixture was boiled for 1 hour at 60°, after which 2 ml of water was added and it was left for 2 hr at room temperature. The mixture was evaporated to dryness and the residue was treated with 15 ml of 9 N ammonia solution and was heated for 1 hr at 60° in a closed vessel and then for 1 hr at 100° under reflux. After filtration, the residue of dicyclohexylurea was washed with 50 ml of water; the filtrate and the wash-waters were evaporated to dryness, and water was added to the residue and evaporated to complete the elimination of ammonia. The residue was dissolved in 10 ml of 85% formic acid and was left overnight at room temperature. 30 ml of alcohol was added and evaporated to dryness; the residue was subjected to evaporation with alcohol twice more and was dissolved in 5 ml of water. 100 mg of barium acetate, ammonia to pH 9, and 20 ml of alcohol were added and the mixture was left overnight in a refrigerator. The precipitate of barium phosphate and the barium salt of the nucleotide were filtered off, washed with aqueous alcohol, and treated on the filter with three 30-ml portions of hot water. The filtrate contained the barium salt of isocytidine-5'-phosphate ($TOD_{246}340$), which corresponds to a 25% yield. The solution was passed through a column (6 × 4 cm) of "Dowex-50" in the morpholinium form and was evaporated to dryness. This gave the morpholinium salt of isocytidine-5'-phosphate, homogeneous on chromatography and paper

electrophoresis.

c. Phosphorylation of N-acetyl-2', 3'-O-isopropylideneisocytidine with 2-cyanoethyl-phosphate and cyclohexylcarbodiimide. A mixture of 21.7 mg (0.067 mmole) of N-acetyl-2', 3'-O-isopropylideneisocytidine and 0.25 ml of a 1 M solution of 2-cyanoethyl-phosphate was dried by distilling off dry pyridine three times, the residue was dissolved in 3 ml of dry pyridine, and the solution was treated with 0.52 g (2.5 mmole) of dicyclohexylcarbodiimide and was heated for 3 hr at 60°. The reaction mixture was treated by method b; the residue after the evaporation of the formic acid was dissolved in 15 ml of water, ammonia was added to pH 9, and the solution was passed through a column (5.5 × 1 cm) of "Dowex" 1 × 4 (200/400 mesh, $HCOO^-$ form). The column was washed with water until the isocytidine had been eliminated, and the isocytidine-5'-phosphate was eluted with a 0.1 N solution of formic acid. The substance was contained in 40-100 ml of eluate, TOD_{255} (pH 1) 180, which corresponds to a yield of 40%. The fractions containing the isocytidine-5'-phosphate were evaporated to dryness and dried in vacuum over KOH. The product was homogeneous on chromatography and paper electrophoresis; from its chromatographic properties and its UV spectrum it was identical with the preparations obtained by methods a and b.

Isocytidine diphosphate glucose

Isocytidine-5'-phosphoromorpholidate (IV). A solution of 100 mg (TOD_{246} 1800 = 0.31 mmole) of the lithium salt of isocytidine-5'-phosphate in 50 ml of water was passed through a column (9 × 1 cm) of "Dowex-50" (pyridinium form); the column was washed with 30 ml of water. The resulting solution of the pyridinium salt was treated with 0.054 ml (0.62 mmole) of morpholine and was evaporated to dryness. The residue of the morpholinium salt of isocytidine-5'-phosphate was dissolved in a mixture of 3.1 ml of water, 3.1 ml of tert-butanol, and 0.054 ml of morpholine, the mixture was heated to the boil, and by means of a linear metering device a solution of 258 mg (1.24 mmole) of dicyclohexyldicarbodiimide in 4 ml of tert-butanol was added over 2 hr. The mixture was boiled for a further 2 hr and was analyzed by paper electrophoresis at pH 7.5. The initial phosphate (R_{UMP} 1.0) was present in only very small amounts, and the reaction product, isocytidine-5'-phosphoromorpholidate, had R_{UMP} 0.48, R_f 0.50 (system 2). The reaction mixture was diluted with 20 ml of water, the dicyclohexylurea was filtered off, and the filtrate was extracted with ether three times to eliminate the dicyclohexylcarbodiimide. The aqueous solution (TOD_{246} 1700) was evaporated to dryness and the isocytidine-5'-phosphoromorpholidate which separated was used without further purification for the pyrophosphate synthesis.

Isocytidine diphosphate glucose (V). The 4-morpholino-N, N'-dicyclohexylcarboxamidinium salt of isocytidine-5'-phosphoromorpholidate (from the preceding experiment) was dried by evaporating off dry pyridine three times and was dissolved in 20 ml of dry pyridine. The solution was treated with 0.90 mmole (9 ml of a 0.1 M solution) of the trioctylammonium salt of α-D-glucose-1-phosphate in 10 ml of dry pyridine and was dried by distilling off dry pyridine three times. The mixture was heated without the access of moisture for 4 hr at 60° and was then diluted with 200 ml of water and extracted with ether (3 × 20 ml). The ethereal extracts were washed with 30 ml of water. The aqueous solutions were combined and passed through a column (10 × 1.5 cm) of DEAE-cellulose (Cl^- form). The column was washed with water (100 ml) to eliminate the pyridine and then with 0.003 N HCl (500 ml; isocytidine-5'-phosphate and diisocytidine-5'-pyrophosphate were eluted, TOD_{246} 490) and 0.003 N HCl + 0.001 N LiCl (500 ml; isocytidine diphosphate glucose was eluted, TOD_{246} 900 = 0.155 mmole = 50% on the isocytidine-5'-phosphate). The fraction containing the isocytidine diphosphate glucose was neutralized with a 10% solution of triethylamine in alcohol to pH 6 and was evaporated to dryness. The residue was dried by distilling off benzene and alcohol (2:1) and was dissolved in 5 ml of methanol, and the lithium salt of the isocytidine diphosphate glucose was precipitated with 35 ml of acetone and 5 ml of ether. The residue was separated by centrifuging and was reprecipitated twice more; it was dried in a desiccator over silica gel, dissolved in water, and converted into the sodium salt on a column of "Dowex-50" (Na form). Lyophilization of the solution gave the sodium salt of isocytidine diphosphate glucose, yield 79.9 mg. The preparation was homogeneous on chromatography and paper electrophoresis, R_f 0.20 (system 2), R_{UMP} 0.90 (pH 7.5), 1.15 (pH 4.0). The nucleoside: glucose ratio after acid hydrolysis was 1:0.90. UV spectrum: in 0.01 N HCl, λ_{max} 220, 256 mμ, λ_{min} 239 mμ; in 0.01 N KOH, λ_{max} 226 mμ. The extinction values per unit weight were found with a preparation 55% pure.

The chromatography and electrophoresis were carried out on paper of the "Goznak" mill; an EFA-1 instrument with a voltage gradient of 20-23 V/cm was used for electrophoresis.

Valuable assistance with the work was given by K. S. Lebedeva.

Summary

1. The synthesis of the isocytidine diphosphate α-D-glucopyranose analog of uridine diphosphate glucose modified at C_2 of the heterocyclic ring has been described.

2. The preparation of nucleotides that are derivatives of isocytidine has been reported for the first time.

REFERENCES

1. N. K. Kochetkov, E. I. Budovskii, and V. N. Shibaev, Izv. AN SSSR, OKhN, 1035, 1962.
2. N. K. Kochetkov, E. I. Budovskii, and V. N. Shibaev, Biokhimiya, 28, 741, 1963.
3. E. I. Budovskii, V. N. Shibaev, G. I. Eliseev, and N. K. Kochetkov, Izv. AN SSSR, OKhN, 1491, 1962.
4. N. K. Kochetkov, E. I. Budovskii, V. N. Shibaev, and M. A. Grachev, Izv. AN SSSR, ser. khim., 1592, 1963.
5. N. D. Gabrielyan, M. A. Novikova, and G. L. Zhdanov, DAN SSSR, 151, 1453, 1963.
6. N. D. Gabrielyan and A. V. Venkina, DAN SSSR, 156, 1379, 1964.
7. N. K. Kochetkov, E. I. Budovskii, V. N. Shibaev, and G. I. Eliseev, DAN SSSR, 159, 605, 1964.
8. N. K. Kochetkov, E. I. Budovskii, V. N. Shibaev, G. I. Eliseev, M. A. Grachev, and V. P. Demushkin, Tetrahedron, 19, 1207, 1963.
9. D. M. Brown, A. R. Todd and S. Varadarajan, J. Chem. Soc., 868, 1957.
10. G. Tener, J. Amer. Chem. Soc., 83, 159, 1961.
11. A. Michelson, J. Chem. Soc., 3655, 1959.
12. W. Koransky, H. Grunze, and G. Munch, Z. Naturforsh, 17b, 291, 1962.
13. J. Moffatt and H. Khorana, J. Amer. Chem. Soc., 83, 649, 1961.
14. S. Rosemann, J. Distler, J. Moffatt and H. Khorana, J. Amer. Chem. Soc., 83, 659, 1961.
15. P. Levene and R. Tipson, J. Biol. Chem., 106, 113, 1934.
16. H. Grunze, Z. anorg. allg. Chem., 296, 63, 1958.

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