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Preparation of Radioisotopically Labelled S-Ribosyl-L-Homocysteine *

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SUMMARY

S-Ribosyl-L-homocysteine radioisotopically labelled in specific moieties has been prepared by enzymatic hydrolysis of the glycosyl bond of sulfur-35 or tritium-labelled S-adenosyl-L-homocysteine. Procedures for chromatographic purification and analysis are reported.

INTRODUCTION.

S-Adenosylhomocysteine is a resultant product of numerous transmethylation reactions involving S-adenosylmethionine [1]. The development of methods for the synthesis of S-adenosylhomocysteine have facilitated studies on the metabolism of this compound. The enzymatic synthesis of S-adenosylhomocysteine from adenosine and L-homocysteine by S-adenosyl-L-homocysteine hydrolase from rat liver provided a convenient method for the production of useful amounts of the pure crystalline compound [2, 3]. Studies on the catabolism of S-adenosylhomocysteine revealed the presence of an enzyme in the cytosol of gram-negative bacteria which cleaved the glycosyl bond of this compound to yield adenine and S-ribosylhomocysteine (Formula 1) [4]. In order to carry out investigations on the metabolism of S-ribosyl-L-homocysteine a method for the preparation of this compound was developed employing the enzymatic cleavage of S-adenosylhomocysteine [4].

This paper reports the method of preparation of S-ribosyl-L-homocysteine radioactively labelled in various positions. Chromatographic procedures used to analyze the preparations are also described.

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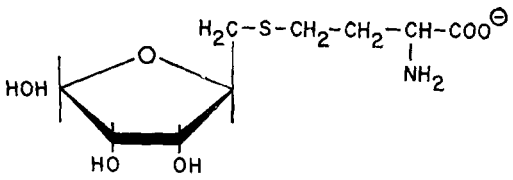

FORMULA I.—S-RIBOSYL-L-HOMOCYSTEINE

MATERIALS AND EXPERIMENTAL PROCEDURE.

L-Homocysteine was prepared from L-homocysteine thiolactone [5]. Adenosine and L-homocysteine thiolactone, uniformly labelled with tritium by the Wilzbach process [6], were obtained from New England Nuclear, Boston, Massachusetts. L-Homocysteine thiolactone-^{35}S was obtained from Volk Isotopes, Skokie, Illinois. The Dowex, Amberlite, and Sephadex resins used were obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey; Mallinckrodt Chemical Works, St. Louis, Missouri; and Pharmacia Fine Chemicals, New York, New York, respectively.

S-Adenosyl-L-homocysteine was enzymatically prepared by the method of Duerre [3] in which crude rat liver extract was incubated with L-homocysteine and adenosine and the resulting S-adenosyl-L-homocysteine purified by ion exchange chromatography and organic solvent extraction. The use of radioactively labelled adenosine or L-homocysteine in the preparation of S-adenosylhomocysteine and subsequent cleavage of this compound by the S-adenosylhomocysteine nucleosidase results in the formation of S-ribosyl-L-homocysteine labelled in different moieties.

Preparation of S-adenosylhomocysteine nucleosidase.

Cell-free extracts. Escherichia coli, strain W, was cultured in four 20-liter carboys containing 15 liters of M-9 medium [7]. To each carboy was added an inoculum of 1 % by volume of a 12-hour culture grown on the same medium. Vigorous aeration was maintained by forcing sterile air through the medium. After 12 hours the cells were harvested by continuous-flow centrifugation at 35,000 × g, washed twice with 0.05 M phosphate buffer, pH 7.8, and resuspended in the same buffer at a concentration of about 400 mg of wet cells per ml. The cells were disrupted in a nitrogen-cooled pressure cell at 26,000 psi and the remaining whole cells and cellular debris were removed by centrifugation at 3.0×10^5 g-min. The extract was incubated with commercial deoxyribonuclease and ribonuclease, 1 mg each per 100 ml of extract, for 1 hour at 37° C. Denatured protein was removed by centrifugation at

1.5×10^5 g-min. Bases, nucleotides, nucleosides, and other small molecular weight materials were removed by passing the extract through a column of Sephadex G-25. A column (4 × 44 cm) previously equilibrated with 4 liters of 0.05 M phosphate buffer, pH 7.8, was suitable for 170 to 200 ml of extract. Elution of the protein using the same buffer was followed with the biruet reagent [8]. This preparation was denoted as Fraction I (Table I).

TABLE I. Purification of S-adenosylhomocysteine nucleosidase

Fraction	Total protein mg	Total activity Units × 10^{-3}	Specific activity Units/mg	Purification Fold	Yield %
I. Crude	6,552	16.9	2.5	1	100
II. Ammonium sulfate, 40-61 % saturated	1,950	12.5	5.9	2.3	74
III. Sephadex G-150	400	6.2	15.6	6.3	37
IV. DEAE-Sephadex-A-50	50	4.0	80.0	32.0	24

Ammonium sulfate fractionation. Ammonium sulfate solution saturated at 0° C containing 1×10^{-3} M EDTA and adjusted to pH 7.0 was added dropwise to the crude extract to 40 %. After mechanical stirring for 15 min, the precipitate was removed by centrifugation at 2.25×10^5 g-min and discarded. The supernatant fluid was raised to 61 % and mechanically stirred for 15 min. The precipitate was collected as before and dissolved in 0.05 M phosphate buffer, pH 7.8, containing 3×10^{-3} M 2-mercaptoethanol to yield a protein concentration of 35 to 50 mg per ml as determined by the method of Warburg and Christian [9] (Fraction II).

Sephadex G-150 gel filtration. Fraction II (20 ml, containing 780 mg of protein) was fractionated on a column of Sephadex G-150 resin as described in Figure 1. S-Adenosylhomocysteine nucleosidase activity and protein concentration were measured on selected tubes to locate the enzyme. The fractions containing the enzyme were pooled and the protein concentrated by the dropwise addition of saturated ammonium sulfate solution to 61 %. The precipitate was collected by centrifugation at 2.25×10^5 g-min, dissolved in the 0.05 M phosphate buffer, pH 7.8, containing 3×10^{-3} M 2-mercaptoethanol, and dialyzed against 4 volumes of the same buffer for 7 hours at 4° C with three changes. This represented Fraction III.

DEAE-Sephadex A-50 fractionation. Fraction III (4 ml, containing 80 mg of protein) was applied to a column of DEAE-Sephadex A-50 resin and fractionated as described in Figure 2. Enzyme activity and protein concentration

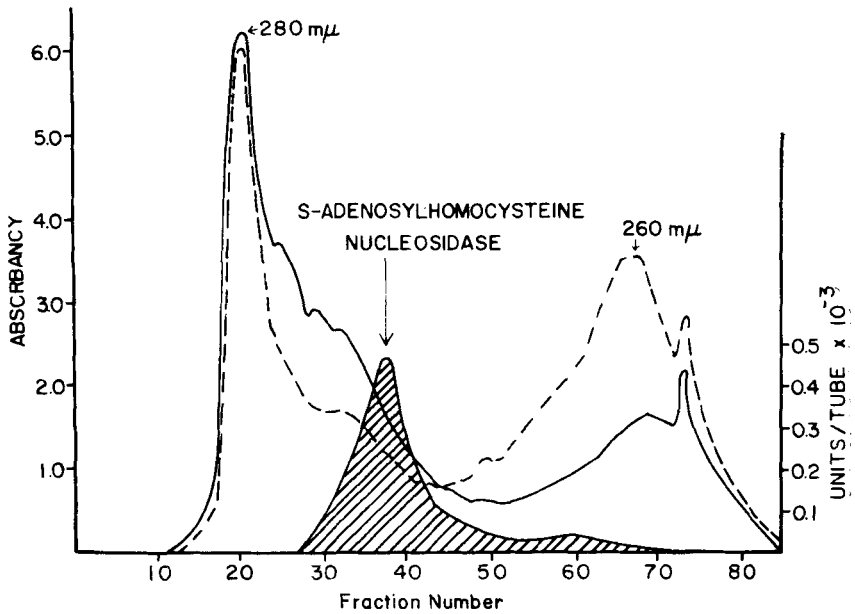

FIG. 1. Gel filtration of S-adenosylhomocysteine nucleosidase on Sephadex G-150 resin. The column, 4×42 cm, was washed with 3 liters of water and equilibrated with 3 liters of 0.05 M phosphate buffer, pH 7.8, containing 3×10^{-3} M 2-mercaptoethanol prior to application of the sample. Elution was performed with the same buffer using a hydrostatic head-pressure of 15 cm and a flow rate of 1.0 ml per min. Sample volumes of 10 ml were collected with the aid of an automatic fraction collector and the elution pattern of the protein was determined spectrophotometrically (9).

were determined on selected 10 ml fractions to locate the enzyme. The fractions containing the enzyme were pooled and used for the preparation of S-ribosyl-L-homocysteine as described under results. This fraction retained 80 % of its total activity after 14 days at —40° C.

Enzyme assay. Reaction mixtures containing 3.0μmoles of S-adenosyl-homocysteine, 100 μmoles phosphate buffer, pH 6.5, and enzyme in a total volume of 1.0 ml were incubated aerobically for 30 min at 37° C. To stop the reaction the tubes were placed in a boiling water bath for 3 min. The method of Benedict (10) was used to determine the amount of reducing compound (S-ribosyl-L-homocysteine) present. Free ribose was used as the standard and gave a linear response in the range of 0.05 to 0.3 μmoles. One unit of activity was defined as that amount of enzyme which catalyzed the liberation of 1.0 μmole of reducing compound per hour under the conditions of the assay.

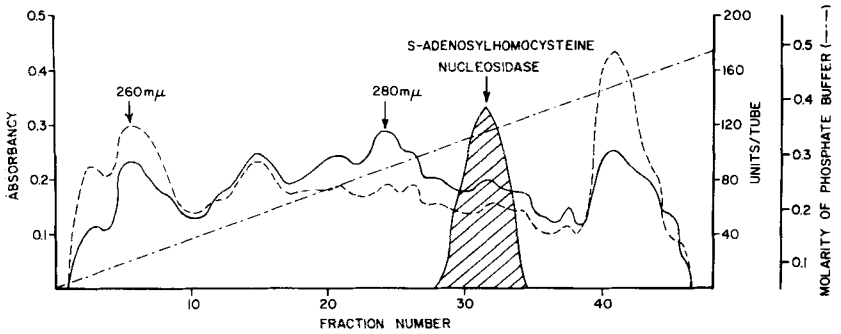

FIG. 2. Fractionation of S-adenosylhomocysteine nucleosidase on DEAE-Sephadex A-50 resin. The column, 2.5 × 18 cm, was equilibrated with one liter of 0.05 M phosphate buffer, pH 7.8, containing 3×10^{-3} M-2-mercaptoethanol prior to application of the sample. Elution was performed by a concentration gradient formed by allowing 250 ml of 0.5 M phosphate buffer, pH 7.8, to flow into a mixing chamber containing an equal volume of 0.05 M phosphate buffer, pH 7.8. Both solutions contained 3×10^{-3} M 2-mercaptoethanol. Elution was followed spectrophotometrically at 260 and 280 mμ small (9).

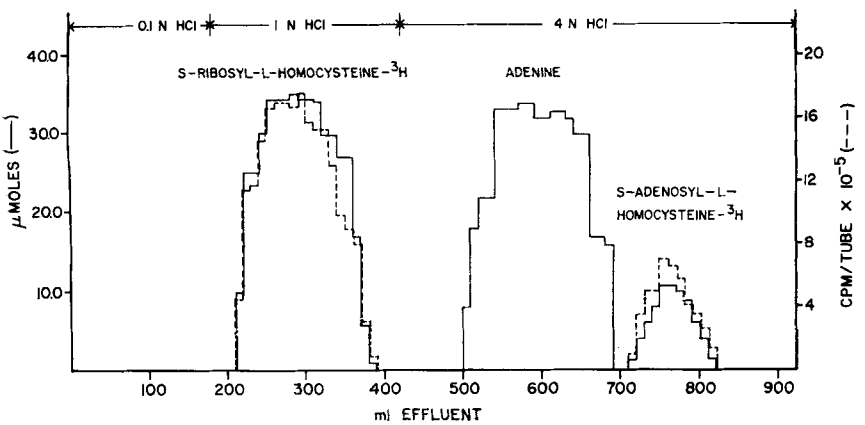

FIG. 3. Purification of S-ribosyl-L-homocysteine-^{3}H. A column of Dowex 50 W- X 8 resin, 200 to 400 mesh, 2.5 × 15 cm, exhibiting a flow rate of 2.0 ml per min was used. The column was washed consecutively with 600 ml of 6 N HCl and 600 ml of 0.1 N HCl prior to application of the sample. Elution was performed as described in the text. The S-ribosyl-L-homocysteine-^{3}H (specific activity of 0.8 mc/ mmole) was determined by the orcinol reaction and scintillation spectrometry, the adenine by ultra violet absorption at 260 mμ, and the S-adenosyl-L-homocysteine-^{3}H (specific activity of 0.117 mc/mmole) by ultra violet absorption at 260 mμ and by scintillation spectrometry.

TABLE II. Incorporation of radioisotopes into various positions of S-ribosyl-L-homocysteine

Substrate used for preparation of S-Adenosylhomocysteine		S-Adenosylhomocysteine		S-Ribosylhomocysteine	
	Specific Activity	Specific Activity	Yield [a]	Specific Activity	Yield [a]
L-homocysteine thiolactone-^{35}S	2.9 mc/mM	0.3 mc/mM	10.3 %	0.28 mc/mM	94 %
Adenosine-^{3}H [b]	153.5 mc/mM	0.98 mc/mM	0.64 %	0.13 mc/mM	13 %
Adenosine-^{3}H [c]	48.0 mc/mM	0.68 mc/mM	1.42 %	0.12 mc/mM	17.6 %
L-homocysteine thiolactone-^{3}H [d]	1.67 mc/mM	0.117 mc/mM	7 %	0.08 mc/mM	67 %

[a] Yield is precent radioisotope recovered in resultant product.
[b] 1,000 mg adenosine exposed to 6 curies of tritium gas for 2 weeks at 26° in water (Wilzbach exchange).
[c] 990 mg adenosine exposed to 3 curies of tritium gas for 2 weeks at 26° in water (Wilzbach exchange).
[d] 980 mg L-homocysteine thiolactone exposed to 3 curies of tritium gas for 1 week (Wilzbach exchange).

RESULTS.

S-Adenosyl-L-homocysteine prepared by the method of Duerre [3] and labelled in the desired position was incubated with the partially purified nucleosidase (Fraction IV, Table I) at a concentration of one μmole per unit of enzyme in the presence of 0.1 M phosphate buffer, pH 6.5, for 2 hours at 37° C. The reaction mixture was deproteinized by heating in a boiling water bath for 4 min, and the clear supernatant fluid obtained by centrifugation at 1.35×10^4 g-min was placed on a column of Dowex 50 W-X8-H^+ resin, 200 to 400 mesh, previously washed with 6.0 N HCl and equilibrated with 0.1 N HCl. The surface area of the column was one cm^2 for each 350 μmoles of S-ribosyl-L-homocysteine. Phosphate and mercaptoethanol were eluted from the column by development with 0.1 N HCl. Approximately 50 ml of acid per cm^2 of cross-sectional area at a column lenght of 14 cm was sufficient. The S-ribosyl-L-homocysteine was then eluted with 1.0 N HCl; approximately 100 ml of acid per cm^2 of cross-sectional area were required. The elution pattern obtained is described in Figure 3. The adenine formed during the reaction and the residual S-adenosylhomocysteine were retained on the column but could be eluted with 4.0 N HCl. The eluate containing S-ribosyl-L-homocysteine was adjusted to pH 6.0 by the addition of Dowex 2-X8-OH^-. This resin was removed by filtration and washed with demineralized water until free of orcinol-positive material. The wash was combined with the filtrate and the solution was lyophylized to dryness and immediately dissolved in water to a desired concentration as determined by the orcinol reaction. Flash evaporation at temperatures below 30° C may be substituted for the lyophilization step; however, this procedure caused a higher precentage of decomposition of the compound. The yields of radioactively labelled S-ribosyl-L-homocysteine ranged from 70 to 80 %.

The degree to which the sulphur-35 and tritiated substrates were incorporated into S-adenosylhomocysteine is described in Table II. The marked reduction in the specific activities of S-adenosylhomocysteine using tritiated substrates might be suspected since considerable radiodecomposition of adenosine or L-homocysteine thiolactone occurs using the Wilzbach procedure. Concern might be expressed as to the purity of the resultant S-adenosylhomocysteine recovered using such substrates directly in the reaction mixtures. However, S-adenosylhomocysteine hydrolase has been found to be quite specific. None of the available nucleotides or purine deoxynucleotides condense with L-homocysteine (Duerre, unpublished data) nor does the D-isomer or oxidation products of L-homocysteine condense with adenosine (ε). The purity of the labelled S-adenosylhomocysteine was accomplished by chromatography on Dowex 50-WX8-H^+ resin, precipitation with phosphotungstic acid, and crystallization. The only impurity detectable by paper chromatography employing butanol-

water-acetic acid (12 : 5 : 3) or ethanol-water-acetic acid (65 : 34 : 1) was S-adenosylhomocysteine sulfoxide [3].

The specific activity of S-ribosylhomocysteine-^{35}S was only slightly less than that of the S-adenosylhomocysteine-^{35}S (Table II). However, a marked reduction in the specific activity of S-ribosylhomocysteine-^{3}H was observed when compared to that of S-adenosylhomocysteine-^{3}H. This reduction may

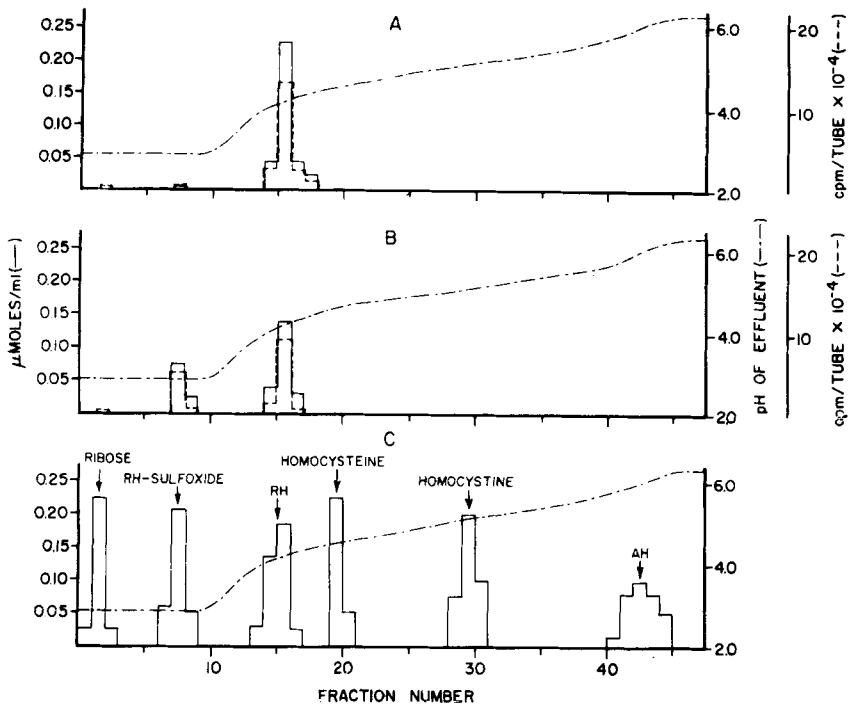

FIG. 4. Chromatography of S-ribosyl-^{3}H-L-homocysteine on Amberlite CG-120 resin. A.—A sample containing 3.5 μmoles of freshly prepared S-ribosyl-^{3}H-L-homocysteine (specific activity of 0.13 mc/mmole) was chromatographed as described in Figure 4 C. B- An equivalent amount of the same sample as described above was stored for 2 weeks at — 20° C at pH 8.0 and chromatographed as described in Figure 4 C. C- Chromatographic separation of ribose, S-ribosyl-L-homocysteine sulfoxide, S-ribosyl-L-homocysteine (RH), homocysteine, homocystine, and S-adenosylhomocysteine (AH). A column of Amberlite CG-120 resin, 200-400 mesh, 0.7 × 20 cm, with a flow rate of 0.6 ml per min was used. The column was washed with 200 ml of 1.0 N NaOH, equilibrated with 0.2 N citrate buffer, pH 2.9, and eluted with a pH gradient formed by allowing 250 ml of 0.2 N sodium citrate, pH 8.3, to flow into a mixing chamber containing an equal volume of 0.2 N citrate buffer, pH 2.9. Both solutions contained 1.0 g Brij-35 per liter. Fractions were collected in 10 ml volumes. Reducing compounds were measured by the orcinol method [11] amino nitrogen by the quantitative ninhydrin test [12] The S-ribosyl-L-homocysteine sulfoxide used as a control was prepared by the method of Toennies and Kolb [13].

have been due to radiodecomposition since S-ribosylhomocysteine-^{3}H had been stored in the crystalline state at —40° C for approximately 1 month prior to use.

When S-adenosyl-^{3}H-L-homocysteine was used only 13 to 17 % of the tritium was recovered in the S-ribosyl-^{3}H-L-homocysteine. The remainder was associated with the adenine. This necessitated the use of S-adenosylhomocysteine with high specific activity in order to obtain S-ribosyl-^{3}H-L-homocysteine with sufficient radioactivity for biological studies.

Analysis of S-ribosyl-L-homocysteine.

Separation of several compounds related to S-ribosyl-L-homocysteine has been achieved by ion exchange chromatography on Amberlite CG-120 resin (Fig. 4). This method has proven to be highly satisfactory in determining the radiochemical purity of the S-ribosyl-L-homocysteine preparations. A typical chromatogram of freshly prepared S-ribosyl-^{3}H-L-homocysteine is presented in Figure 4A. The only radiochemical impurities found were S-ribosyl-^{3}H-homocysteine sulfoxide and an unidentified compound which passed directly through the column. These two impurities constituted less than 5 % of the total radioactivity recovered. The compound could be stored at —40° C under slightly acidic conditions (pH 5.5 to 6.5) for approximately 2 weeks without significant increase in oxidation or decomposition products. Similar results were obtained from the chromatographic analyses of S-ribosyl-L-homocysteine-^{3}H and S-ribosyl-L-homocysteine-^{35}S with specific activities of 0.08 and 0.28 mc/mmole, respectively.

When aliquots of the S-ribosyl-^{3}H-L-homocysteine preparation were adjusted to pH 8.0 and allowed to stand at room temperature or at —40° C for more than 3 days, oxidation to S-ribosylhomocysteine sulfoxide occurred (Fig. 4B). No increase in the amount of the unidentified radioimpurity resulted.

Discussion.

The procedure described for the incorporation of radioisotopes into specific moieties of S-ribosyl-L-homocysteine has greatly facilitated investigations concerning the metabolism of this compound [14, 15]. The specific activities and radiopurity (95 %) of the S-ribosyl-L-homocysteine preparations were more than adequate for radioisotopic assay of small quantities of the compound present in biological materials, and the trace amounts of radiochemical impurities found in these prepatations have not greatly interferred with these assays. The type of investigation to be performed using S-ribosyl-L-homocysteine is a criterion for the amount of purity required of the preparations. If the demands on purity are not great then the S-adenosylhomocysteine nucleosidase enzyme

used for the formation of S-ribosyl-L-homocysteine from S-adenosyl-homocysteine may be purified only through the steps of ammonium sulfate or Sephadex G-150 fractionation. However, it has been shown that these fractions contain small amounts of an enzyme which cleaves the thioether linkage of the S-ribosyl-L-homocysteine yielding free homocysteine [14, 15]. The presence of this enzyme in the nucleosidase fractions results in trace amounts of free homocysteine in the final S-ribosyl-L-homocysteine preparations. The nucleosidase purified through the DEAE-Sephadex A-50 fractionation was free from detectable amounts of the S-ribosyl-L-homocysteine cleavage enzyme.

The formation of S-ribosylhomocysteine sulfoxide from S-ribosyl-L-homocysteine resulted from chemical oxidation, but the amount of oxidation during the actual preparation was found to be negligible resulting in only trace amounts of the sulfoxide. However, if the preparations were kept in the dry state in the presence of air, oxidation occurred rapidly with 40 % loss of the S-ribosyl-L-homocysteine over night. The formation of the sulfoxide was also enhanced under alkaline conditions but could be kept at a minimum during storage if particular care was taken to keep the pH of the final aqueous preparations between 5.5 and 6.5.

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