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NOTE

Modification of the ^{3}H-leucine centrifugation method for determining bacterial protein synthesis in freshwater samples

A. K. T. Kirschner, B. Velimirov*

Institut für Medizinische Biologie, Arbeitsgruppe Allgemeine Mikrobiologie, Universität Wien, Währingerstr. 10, A-1090 Wien, Austria

ABSTRACT: The validity of the ^{3}H-leucine centrifugation method for determining bacterial secondary production in oligotrophic and eutrophic fresh- and seawater samples was examined. For freshwater samples, we found that the established protocol developed by Smith & Azam (1992) led to significantly lower values (up to 57%) than a novel protocol presented here, where bacterial proteins are precipitated under acidic conditions (trichloroacetic acid) at 4°C with a humic extract and solubilizing DNA and RNA at 100°C for 30 min. For seawater samples, no difference was found when an ethanolic washing step was included in the novel protocol. We also used different salt solutions instead of humic extract; these both act as co-precipitants for the precipitation of the proteins. An unbuffered 3.5% (final conc.) NaCl solution was found to be highly effective and gave consistent results and lower blank values. Incorporation rates obtained with our protocol showed good agreement with the commonly used filtration method. Therefore, we argue that for freshwater samples an NaCl or humic extract addition is necessary for an efficient precipitation of the proteins when the centrifugation method for determining bacterial secondary production via ^{3}H-leucine incorporation is applied.

KEY WORDS: ^{3}H-leucine · Centrifugation · Filtration · Protein · Bacterial secondary production

Several approaches based on the incorporation of radioactive substances into bacterial biomass for estimating bacterial secondary production rates have been presented during the past 15 yr, since the introduction of the ^{3}H-thymidine method by Fuhrman & Azam (1980). The method currently used in most studies is the measurement of bacterial protein production via incorporation of radioactive leucine (Kirchman et al. 1985, Simon & Azam 1989). To separate the labeled proteins from other macromolecules and from unincorporated label, filtration through cellulose membrane filters (Sartorius, Millipore) with nominal pore-sizes of 0.1 to 0.45 µm are used. In 1992, Smith & Azam proposed a novel protocol for estimating bacterial protein production via incorporation of ^{3}H-leucine, separating the bacterial proteins by centrifugation rather than by filtration. This centrifugation method was shown to be faster, much cheaper and more effective than the filtration method and it produced less radioactive waste. Before adapting the centrifugation method for the above-mentioned reasons, we compared both methods using samples from a eutrophic freshwater pond and an oligotrophic alpine lake. The filtration approach, performed after the protocol of Simon & Azam (1989), which has also been routinely used in our lab, led to significantly higher values than the centrifugation approach proposed by Smith & Azam (1992). We therefore developed an improved centrifugation protocol by utilizing humic substances as co-precipitants for the macromolecule precipitation, an approach which was routinely applied in studies on the determination of bacterial production rates in sediments (e.g. Moriarty & Pollard 1982, Kaplan et al. 1992). In this context we tried to answer 2 questions: first, whether the application of humic substances would also improve the protocol for the analysis of saltwater samples and, second, whether a methodological analysis of the applied procedure would indicate if alternative co-precipitants could be used instead of humic substances.

Materials and methods. ***Sampling:*** Freshwater samples were collected with a Schindler sampler from the Alte Donau, a eutrophic backwater creek of the river Danube in Vienna, from the Kühwörter Wasser (mesotrophic), from the Neue Donau (mesotrophic), and from the Lunzer Untersee, an oligotrophic alpine lake (all in Austria), and brought to the laboratory at *in situ* temperature within 60 min. Oligotrophic seawater from the Bay of Calvi, Corsica, France, was sampled with sterile 1 l glass bottles at STARESO (Station de Recherches Sousmarines et Oceanographiques); eu-

*Addressee for correspondence.
E-mail: branko.velimirov@univie.ac.at

trophic seawater samples were from the Gulf of Trieste near Sistiana, Italy. Incubations were started immediately after collection of the samples.

Centrifugation method: 1 ml of sample was dispensed into 1.7 ml screw cap microcentrifuge tubes (Eppendorf). 10.7 µl of ^{3}H-leucine (NEN research products) with a specific activity of 6.6 TBq mmol^{-1} were added to the tubes to achieve a final concentration of 60 nM in the case of the eutrophic and mesotrophic fresh- and seawater samples, and 3.6 µl in the case of the oligotrophic samples (20 nM final conc.). Incubations were stopped after 30 min with 60 µl of 100% trichloroacetic acid (TCA) to yield a final concentration of 5%. All incubations were run in 6 replicates and 3 blanks, to which TCA was added prior to the addition of the isotope.

For optimal precipitation of the proteins, 50 µl of a humic extract (preparation see below), acting as a co-precipitant and allowing visualization of the pellet, were added and the macromolecules were precipitated at 4°C for 30 min. The samples were then boiled for 30 min in a waterbath to solubilize DNA and RNA. For freshwater samples, as an alternative to the humic extract, 150 µl of a tris-buffered saturated ammonium sulfate solution (SAS) was added, which is often routinely used for salting out proteins (Ausubel et al. 1989). Tris-buffered sodium chloride (3.5% final conc.) was also tried as co-precipitant. As the addition of both salt solutions resulted in even lower values than the protocol of Smith & Azam (1992), salt solutions were prepared without buffer to prevent buffering of the TCA. Proteins were precipitated at 18°C for 30 min.

After centrifugation at 14000 rpm (15800 × *g*) for 5 min (Eppendorf 5415C), the supernatant was decanted and the samples were washed with 1 ml of 5% TCA. Ethanol (80%) was also used for the washing step instead of 5% TCA in order to determine whether a significant portion of the tritium label occurred in the lipid fraction (Wicks & Robarts 1988, Hollibaugh & Wong 1992). In this step the samples were vortexed vigorously after the addition of the ethanol to avoid inefficient extraction of the lipids.

To redissolve the proteins from the pellet, 200 µl of 1.2 N NaOH were added and boiled for 20 min in a waterbath. 1.3 ml liquid scintillation cocktail (Ultima Gold; Canberra Packard) were then added to the Eppendorf tubes. The tubes were put into 20 ml scintillation vials and radioactivity was determined with a Canberra Packard liquid scintillation counter (1900 TR). Counts were automatically corrected for quenching using a stored standard curve and a machine counting efficiency program. Initially, we tried to measure the radioactivity in the proteins by adding the scintillation cocktail directly into the centrifugation tube, as proposed by Smith & Azam (1992), but the counts achieved with this procedure were only 18% (SD: 3%) of the counts achieved after boiling with 1.2 N NaOH (data not shown) for the samples precipitated with humic extract as co-precipitant. Using other cocktails from Canberra Packard (Lumasafe and Lumasafe^{+}) also led to unsatisfying results with counts being 68% (SD: 4%) and 50% (SD: 8%) lower than those obtained after boiling with NaOH. Even with the cocktail Ecoscint (National Diagnostics) used by Smith & Azam (1992), the achieved counts were 76% lower. This was due to the influence of the humic substances, which prevented a complete dissolution of the precipitated proteins by the cocktail. When humic substances were omitted, the mentioned cocktails achieved 105% (SD: 4%), 89% (SD: 6%), and 87% (SD: 8%) of the counts measured after boiling with NaOH (data not shown).

The data obtained by the protocol described above were compared to data resulting from the method proposed by Smith & Azam (1992) for seawater samples. In this method the extraction of the labeled proteins was performed with TCA (5% final concentration) at a temperature of 18°C for 30 min without adding a humic extract as a co-precipitant, followed by centrifugation and a washing step with 5% TCA. No boiling step was included for the removal of label in the DNA fraction. The precipitated proteins were also dissolved in 1.2 N NaOH instead of adding the scintillation cocktail directly to the microcentrifuge tubes. In all cases where NaOH was used in combination with scintillation cocktails care was taken to control for possible chemoluminescence effects.

Preparation of a humic extract: Instead of soil (e.g. Moriarty & Pollard 1982, Kaplan et al. 1992), 20 ml of the silty sediment of the Alte Donau were boiled for 1 h in 80 ml of a NaOH (0.6 N)/SDS (0.1%)/EDTA (0.05 M) solution to extract humic substances, DNA and proteins. The extract was then filtered through a Whatman GF/F filter to obtain a particle-free solution.

Filtration method: We followed the protocol of Simon & Azam (1989) to determine the uptake of ^{14}C-leucine into bacterial proteins. Six 10 ml replicates and 3 blanks were put into sterile 20 ml polyethylene scintillation vials (Canberra Packard). 2.1 µl of ^{14}C-leucine (NEN research products; specific activity: 12.0 GBq mmol^{-1}) were added to the eutrophic samples to reach a final concentration of 60 nM; oligotrophic samples received 0.7 µl of ^{14}C-leucine corresponding to a final concentration of 20 nM. 500 µl of TCA were added to the tubes which served as blanks immediately before the addition of the label. Incubations were terminated with 500 µl TCA and the proteins were extracted by boiling the samples for 30 min at 100°C. After cooling, the extracted samples were filtered onto 0.1 µm pore-size cellulose membrane filters (Sartorius) and rinsed with 5 ml of distilled water. As in the centrifugation

method, we tried to optimize the protein extraction process by chilling the samples for 30 min at 4°C prior to the boiling step. In addition, in some experiments 80% ethanol was used for rinsing the filters instead of distilled water. One incubation series was performed with samples from the Alte Donau to check for possible differences in calculated uptake rates when ^{14}C-leucine or ^{3}H-leucine is used.

Statistical analysis: Comparison of the data was performed using Student's *t*-test.

Results and discussion. The data obtained by following the protocol described here (Fig. 1, pathway A) were compared to the data obtained by the method described by Smith & Azam (1992). For the eutrophic Alte Donau, on May 15, 1997 (Fig. 2A), the established centrifugation method (Smith & Azam 1992) yielded 57% lower values than the incorporation rates obtained by the method presented here ($p < 0.001$), and on July 15, 1997, the difference amounted to 20% ($p < 0.02$; Fig. 2B). The same was observed for samples from the oligotrophic lake with a difference of 23% ($p < 0.02$; Fig. 2C). Washing with 80% ethanol instead of 5% TCA in order to remove label associated with the lipid fraction did not result in significantly lower values ($p > 0.4$). Since boiling the samples in hot TCA removes label occurring in DNA and RNA fractions, one would expect that extracting the samples on ice (4°C) for 30 min in combination with a co-precipitant (humic extract) would lead to a more effective precipitation of the proteins. Moreover, the visualization of the pelleted material avoids erroneous withdrawal of precipitated proteins during removal of the supernatant. One effect of the humic substances was an increase in the blank values, but the coefficients of variation of the blanks obtained by the method of Smith & Azam (1992) were similar to the ones obtained with our protocol for each ecosystem (data not shown).

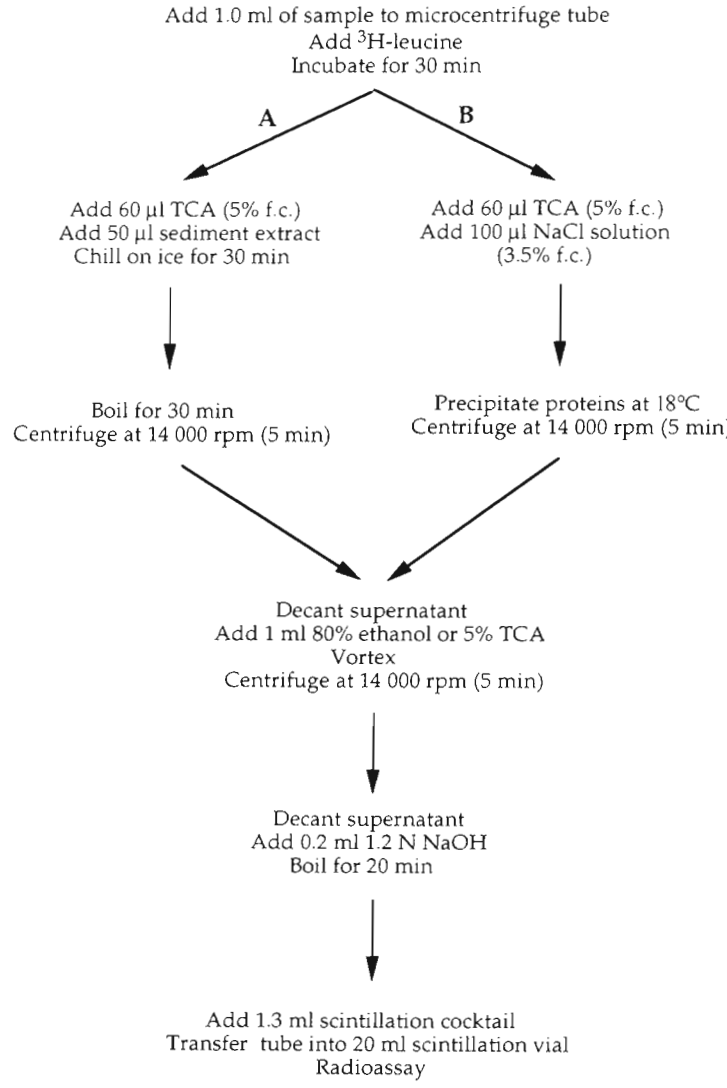

Fig. 1 Two novel protocols for the determination of bacterial ^{3}H-leucine uptake via the centrifugation method, using humic extract (pathway A) or NaCl (pathway B) as co-precipitants

The data obtained by the centrifugation method were also compared to parallel measurements made with the filtration method (Fig. 3) for the eutrophic freshwater samples. These investigations were performed on 3 occasions at 2 stations of the Alte Donau. On average the values obtained with the centrifugation method were 14% (SD: 2.5%) higher than the values from the filtration method. Precipitating the macromolecules for 30 min on ice prior to boiling was performed for the filtration method on 2 occasions (Fig. 4). On August 12, 1997, this procedure led to values 13% higher than those obtained without this additional step; on August 26, 1997, 17% higher values were observed. However, because of the high standard deviations, the observed difference was not statistically significant. Rinsing the filters with 80% ethanol had no significant effect on the results, but resulted in higher blank values. On May 22, 1997, we checked if the isotope had an influence on the calculated leucine uptake rates. We found no difference between samples supplemented with ^{14}C-leucine or with ^{3}H-leucine ($p > 0.5$; $n = 6$).

Comparison between the 2 centrifugation methods mentioned showed that for both the oligotrophic and the eutrophic marine samples, unlike for the freshwater samples, the protocol described by Smith & Azam (1992) did not lead to significantly lower values than the method proposed in this study (Fig. 2D,E; $p > 0.05$), when the samples were washed with 80% ethanol. Smith & Azam (1992) had observed no effect caused by an ethanolic washing step, which we were able to

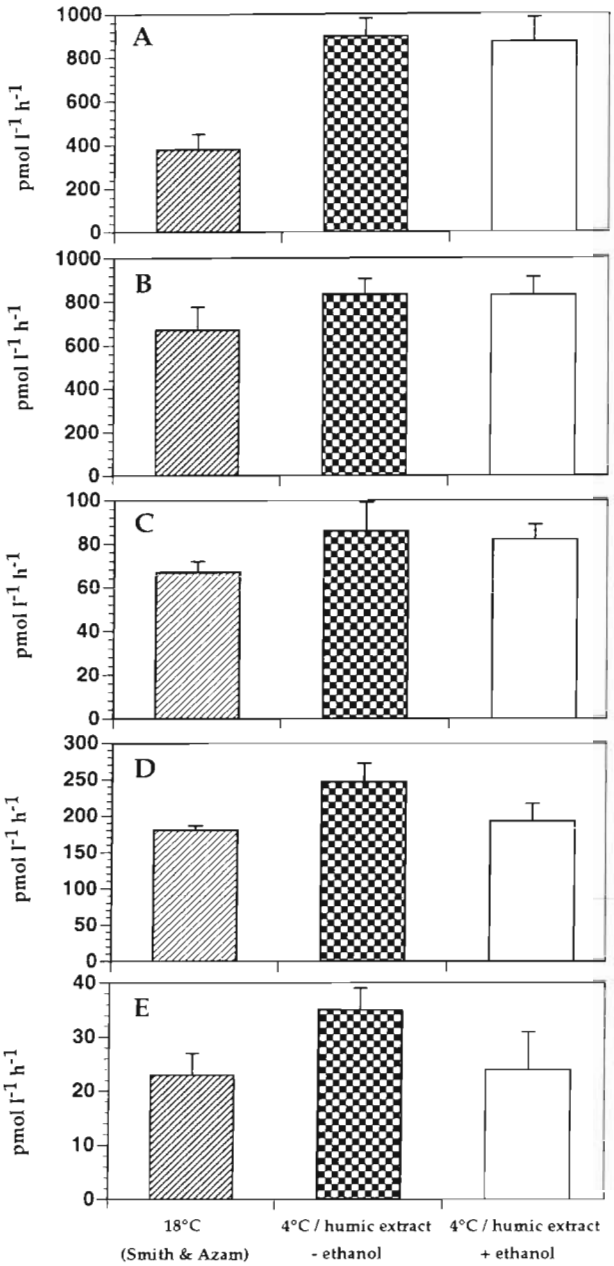

Fig. 2. Comparison of bacterial ^{3}H-leucine uptake rates determined by the different centrifugation protocols. (A, B) Alte Donau (May 15, 1997, and July 15, 1997); (C) Lunzer Untersee (May 8, 1998); (D) Gulf of Trieste (May 9, 1998); (E) Bay of Calvi (July 10, 1997). Bars represent mean of 6 replicates + 1 standard deviation

prove with samples from both fresh- and seawater (data not shown). Therefore we assume that certain lipid fractions may not precipitate at 18°C while they do so at 4°C, as shown above. Also, no statistically significant difference was detected between the centrifugation and the filtration method for the saltwater samples (data not shown).

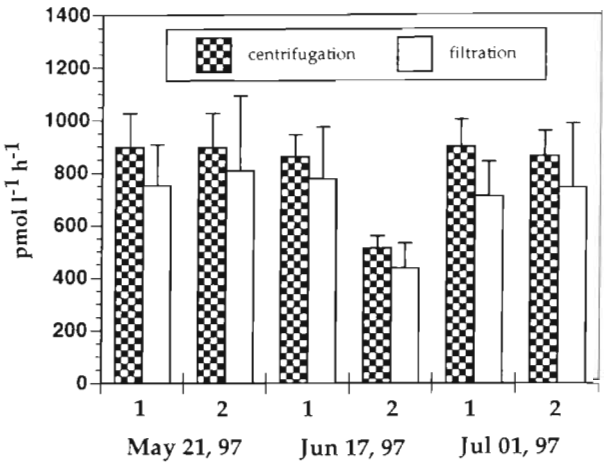

Fig. 3. Comparison of bacterial ^{3}H-leucine uptake rates, determined by the filtration method and the centrifugation method presented here, on 3 occasions and 2 sampling sites (1, 2) in the Alte Donau. Bars represent mean of 6 replicates + 1 standard deviation

Because the addition of a humic extract did not lead to different production estimates than those obtained by the protocol of Smith & Azam in marine water samples, and because salting out is known to be a common procedure for precipitating proteins in molecular biology studies (Ausubel et al. 1989), it was assumed that precipitation conditions were close to optimal in marine water samples. Consequently, we questioned whether special salt species could be used as an alternative to the humic extract for the freshwater samples. First we tested tris-buffered solutions of saturated ammonium sulfate (SAS; Ausubel et al. 1989) and sodium chloride (3.5% final conc.). As the addition of

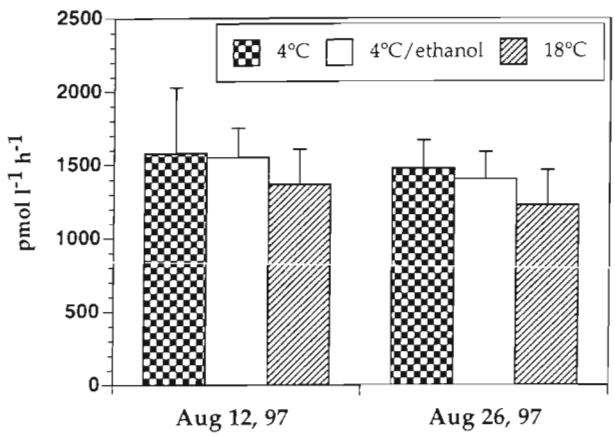

Fig. 4. Comparison of bacterial ^{3}H-leucine uptake rates, determined with different protocols of the filtration method, on 2 occasions in the Alte Donau. Bars represent mean of 6 replicates + 1 standard deviation

both salt solutions resulted in even lower values than the protocol of Smith & Azam (1992) (Fig. 5A), salt solutions were prepared without buffer to prevent buffering of the TCA. For all 3 investigated freshwater sites the protocol using unbuffered NaCl (Fig. 5B) led to the same results as the protocol with humic extract and additionally resulted in lower blank values. Thus, NaCl can be recommended as an alternative co-precipitant to humic extracts (Fig. 1, pathway B). SAS, on the other hand, gave more inconsistent results than NaCl and had lower values and higher standard deviations (Fig. 5B).

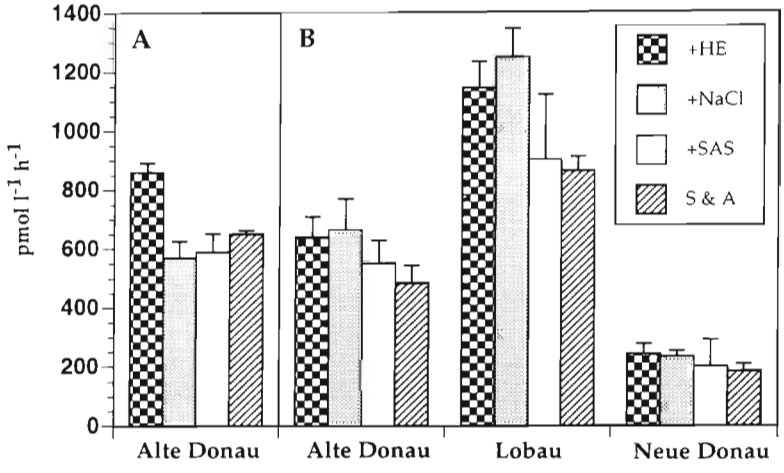

Fig. 5. Comparison of bacterial ^{3}H-leucine uptake rates, determined with different protocols of the centrifugation method from 3 freshwater ecosystems in different trophic states. (A) Tris-buffered solutions of sodium chloride (NaCl) and saturated ammonium sulfate (SAS) were used. (B) Salt solutions were prepared without buffer; HE: humic extract; S & A: Smith & Azam (1992). Bars represent mean of 6 replicates + 1 standard deviation

As already mentioned above, we tried different scintillation cocktails to dissolve the precipitated proteins directly in the centrifugation vials, but the highest values were always provided by redissolving the proteins in NaOH. Chemiluminescence, which is sometimes observed with basic substances in combination with scintillation cocktails and which leads to higher counts, could be ruled out for several reasons. First, chemiluminescence causing counts in the energy range from 0 to 2 keV is automatically detected and substracted by the Canberra Packard scintillation counter. Second, the ratio between detected counts in the energy window from 0 to 2 keV and from 2 to 156 keV was the same for samples with and without NaOH (data not shown), and third, no difference was observed between samples measured 20 min and 24 h after the addition of the cocktail.

Conclusions. For all investigated freshwater samples the method proposed by Smith & Azam (1992) led to significantly lower values than our protocol described here. The effect of both precipitation of proteins at 4°C instead of 18°C and of using a humic extract as co-precipitant resulted in a much more efficient precipitation of the labelled proteins. Boiling of the samples was necessary to solubilize DNA and RNA, which co-precipitate at a temperature of 4°C. An ethanolic washing step to remove labelled lipids showed no effect on the measured incorporation rates, but for the samples from seawater a reduction of 36 and 22% was observed, leading to values equal to those obtained by the established method of Smith & Azam (1992). This shows that the method established by Smith & Azam can be applied for seawater samples, but is not reliable for freshwater samples. Utilization of a NaCl solution (3.5% final conc.) as an alternative to the humic substances can be highly recommended, as it resulted in consistent incorporation rates and low blank values. In addition, NaCl solutions can be prepared much more easily than a humic extract and the protocol becomes less time consuming.

In general, the rates obtained with our centrifugation protocol were in good agreement with the results from the filtration method. For the freshwater samples the centrifugation method led on average to values 14% higher than the filtration method. Precipitation of the proteins at 4°C prior to the boiling step resulted in 13% to 17% higher values than the filtration approach proposed by Simon & Azam (1989). This increase was of the same magnitude as the difference between the filtration and the centrifugation method and could explain this observed difference. For the seawater samples no significant difference between the centrifugation and the filtration approach was observed.

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