塩誤差を伴わない水中微量アンモニアの直接定量法

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A Direct Estimation of Microgram Amounts of Ammonia in Water without Salt-error

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A direct colorimetric method has been developed for the determination of ammonia in water without salt-error by using the phenol-hypochlorite reaction. As a large amount of potassium carbonate was added to make the condition of indophenol blue formation nearly constant without regard of the salinity of samples, the method could be made free of salt-error in the determination of samples of either fresh or sea water whose chlorinity was less than 28‰. BEER's law was applicable up to a concentration of 0.6 ppm NH_3-N, and the standard deviation of each determination was about 2.2 ppb N irrespective of the concentration. The amount of reagents and the conditions of the procedure were examined in detail. Twenty-five inorganic and thirty-eight organic compounds, including twenty-one L-amino acids, were tested for interference, and little interference was observed in the not strongly polluted samples.

Among many methods available for estimating ammonia in sea water[1-15], only a few methods are of use for applying to littoral or estuarine water of varying salinity from aspects of either feasibility or sensitivity. The feasible methods that have high sensitivity are either RICHARDS-KLETSCH's[15], YOSHIDA's[14] or modified LOBOCHINSKY-ZALTA's methods[5,10,11] which do not employ separation of ammonia or the derivatives from sea water with either distillation[4], diffusion[6] or solvent extraction[7-9]. RICHARDS-KLETSCH's method employs different working reagents for fresh water and for sea water, and detects other labile nitrogenous compounds such as many amino acids.[15] YOSHIDA's method detects urea and is troublesome to cultivate and to maintain the slowly growing bacterium which converts ammonia to nitrite[14]. On the other hand the modified LUBOCHINSKY-ZALTA's methods which adopt phenol-hypochlorite reaction detect only a few nitrogenous compounds other than ammonia, and the color developed is stable[5,10,11,22]. But as the modified methods already reported have salt-error[5,10,11], they are not convenient to be applied to the samples from littoral or estuarine water whose salinity varys significantly with time and/or locations. If LUBOCHINSKY-ZALTA's method is modified to eliminate salt-error, it will become very convenient and can be applied to study the nitrogen excretion from aquatic animals.

The great difference between fresh and sea water is that of buffer capacity and of ionic concentration. The optimal pH for color-development in LUBOCHINSKY-ZALTA's method was reported either about 10[5], 11[10,22] or 12[16,17], where some cations in sea water

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usually precipitated. After preliminary study on the buffer systems of carbonate and of phosphate, K_2CO_3–Na_2HPO_4 buffer system was examined to eliminate the salt-error from the colorimetry. While making the ionic concentration nearly constant without regard of salinity of sample, the addition of large amount of potassium carbonate can bring the pH of colored solution nearly to the optimum, and make the final volume of colored solution increase little because of the extreme solubility of potassium carbonate. The addition of dibasic sodium phosphate is expected to reduce the pH against the addition of large amount of potassium carbonate so as to adjust the pH of colored solution to the optimum, and also makes the precipitation thus formed settle fast. Employing such a buffer system, preliminary experiments showed the salt-error could be almost eliminated. The elimination of salt-error enables the easy calibration and dilution with deionized water, which is very important for routine works. An investigation was made to know the optimal addition of other reagents and the optimal condition for color-development.

Experimental

Apparatus A tube mixer, preferably of vortex type. Two automatic dispensing pipettes for reagents F and G, or measuring pipettes (5 and 10 m*l* capacities) with a rubber safty-pipetter which is commonly used in radio-isotope laboratories. A temperature-controlled water bath (70–75°C). A spectrophotometer or colorimeter with a 625 nm filter, and 1 cm glass cells.

Reagents All the chemicals used in this work were the guaranteed grade ones of the Japanese industrial standard as far as they were standardized. **Phenol solution (A)** Dissolve 60 g of phenol, 3.0 g of glacial acetic acid* and 1.36 g of sodium acetate ($CH_3COONa \cdot 2H_2O$)* in 1000 m*l* of deionized water. Stable for several months if stored in a dark stoppered glass bottle at refregerator temperature. **Nitroprusside solution (B)** Add 3.0 g of sodium nitroprusside (sodium nitroferricyanide, $C_5FeN_6NaO \cdot 2H_2O$) to 100 m*l* of deionized water. Stable for several months in dark. **Carbonate buffer (C)** Dissolve 900 g of K_2CO_3 and 60 g of $Na_2HPO_4 \cdot 12H_2O$ in 1000 m*l* of deionized water. The volume of the solution amounts to about 1.3 *l*. Stable in a polyethylene bottle indefinitely. **Hipochlorite solution (D)** Dilute commercial sodium hypochlorite (antiformin) with either distilled or deionized water to make the concentration of effective chlorine 5.0 percent after knowing the strength with iodometric titration. Stable for several months if stored as reagent A. **Stock standard ammonia solution (E)** Dissolve 0.4716 g of ammonium sulfate in distilled water to make the volume 1000 m*l*. Stable for at least six months with addition of 1 m*l* of xylene as antiseptics if stored as reagent A. **Phenate mixture (F)** Mix reagent A with B (100: 2, v/v). Store in a dark glass-stoppered

* When dealing with usual fresh and sea water samples, the acetate buffer may be omitted without any trouble, but some error is expected in the determination of samples of either high or low pH value.

bottle. Prepare on the day of use. **Hypochlorite mixture (G)** Mix reagent C with D (100: 5, v/v). Prepare on the day of use. **Working standard ammonia solution (H)** Dilute reagent E with deionized water to make the concentration of 0.4–0.6 ppm of ammoniacal nitrogen according to the colorimeter. Prepare on the day of use.

Procedure Mix 1 m*l* of reagent F with 10 m*l* of sample (0–0.6 ppm NH_3–N) in a test tube (18×180 mm) thoroughly with a tube mixer. Then add 2 m*l* of reagent G (sp. gr. 1.5), and immediately mix completely homogeneously. After standing at room temperature for about thirty minutes, put the tubes in a water bath of temperature of 70–75°C and leave there for about thirty minutes. Then cool to room temperature. After breaking down the flocks of precipitation if any with a tube mixer, stand the tubes for more than four hours to settle the precipitation. Or centrifuging may be employed if necessary. Measure the absorbance of supernatant solution in a 1 cm glass cell at the wave length of 625 nm against distilled or deionized water. For the calibration it is desirable to develop the color of deionized water and of working standard solution (H) in each run, as the reaction is sensitive to temperature.

Results and Discussion

Amount of reagents From preliminary experiments reagents A and C were selected for their solubility and buffer capacity. Then other reagents were tested. The amount of reagents and the condition of analyses were same with that described above unless mentioned. Ammonia-free sea water was prepared on the day of use by the following method as described by MANABE[10]: MF-filtered sea water was diluted with the same amount of deionized water, and the ammonia was boiled out by bubbling with base- and acid-washed air under reduced pressure at the temperature of 70°C to make the water to its original volume. The ammonia content of the water thus obtained was reduced to one over one hundred and twenty, and that of original sea water was usually less than 50 ppb of ammoniacal nitrogen. pH of the water thus obtained was more than 9.0. Chlorinity of the water was determined by SARUHASHI's method[18].

When 0.5–2.0 m*l* of reagent F was added, addition of 1.0–2.0 m*l* gave the same maximal color development for both deionized and sea water samples. Addition of 2 m*l* did not increase the range to which BEER's law was applicable.

When the ratio of reagent B to A was varied from 0.33 to 6.67 percent, the ratio of 1.33 to 6.67 percent gave the same maximal color development for both deionized and sea water samples.

When 1–3 m*l* of reagent G was added, addition of 1.0–2.5 m*l* gave the same maximal color development for both deionized and sea water samples. The more addition of reagent G was expected to increase the applicable range of salinity because of the stronger buffer capacity, though slight decrease in absorbance was observed with increase in the

addition.

When the ratio of reagent D to C was changed from 1.25 to 15 percent, the ratio above 2.5 percent gave the same color development for both deionized and sea water samples, but the sensitivity decreased with increase in the ratio.

Stability of working reagents As phenol was prone to redden on exposure to air and light, and as sodium nitroprusside slowly decomposed, the stability of reagent F was studied as shown in Table 1. The sensitivity did not change during 0–6 (22) hours after making reagent F, but the blank absorbance increased a little ($0.6–1.2 \times 10^{-3}$ Abs./h). But direct sunlight deteriorated the reagent very quickly.

Reagent G was stable in respect to the concentration of effective chlorine for at least 19 hours after made, even if it was kept at the temperature of 33°C.

Table 1. Stability of reagent F.

Ammonia-N, ppm	Time after making reagent F, min. 2.5	15	30	60	120	225	330	1340	Laboratory temperature, °C
	Absorbance								
0.4	.439	.444	.441	.443	.440	.443	.444	.469	9
0	.034	.034	.033	.034	.035	.036	.036	.048	
0.4	.442	.439	.443	.441	.443	.443	.441	.466	29
0	.013	.010	.010	.011	.013	.016	.018	.037	

Deionized water was used for basal medium. Reagents and samples were kept at laboratory temperature.

Intervals among mixing reagents, heating and colorimetry The interval between mixing reagents F and G did not affect the sensitivity at least up to 2.5 hours as shown in Table 2, but the blank absorbance increased a little.

As shown in Table 3 the interval between mixing reagent G and heating did neither affect the sensitivity nor the blank absorbance when it was 15–280 minutes after mixing.

Once the color fully developed, fading was very little, 1.5–4.0 percent per week, even if the tubes were placed on a desk without stopper. Even if the colored solution was contaminated with ammonia, the color did not change when the pH of solution did not vary.

Temperature of laboratory The sensitivity increased when treating samples at a high temperature as shown in Tables 1 and 2. In the room of greatly fluctuating temperature it is recommended to keep both samples and working reagents in a water bath of temperature near to the room temperature.

Heating for color-development Table 4 shows the effect of temperature of water bath on the color-development. The temperature in tube may vary with the ratio of the heat capacity and/or heating power of water bath to the number of tubes placed in. As the time of heating should not be chosen too short without regard of the time

constant of the system, 30 minutes of heating at temperature of 70–75°C was employed to heat all the tubes equally. Above the temperature the color faded within one hour, and below the temperature it required too much time to develop the color.

Table 2. Effect of interval between additions of reagents F and G.

Ammonia-N, ppm	Interval, min. 0.8	6.5	14	27	45	75	111	150	Laboratory temperature, °C
	Absorbance								
0.4	.422	.421	.423	.421	.417	.417	.421	.423	9
0	.010	.010	.010	.011	.010	.009	.011	.011	
0.4	.460	.462	.456	.458	.469	.464	.472	.478	29
0	.020	.021	.022	.024	.024	.026	.027	.028	

Deionized water was used for basal medium. Reagents and samples were kept at laboratory temperature.

Table 3. Effect of interval between addition of reagent G and heating.

Ammonia-N, ppm	Interval, min. 0	2.5	15	30	45	60	120	180	240	280
	Absorbance									
0.4	.428	.433	.445	.447	.447	.447	.445	.443	.445	.443
0	.015	.015	.015	.015	.015	.015	.015	.015	.015	.015

Deionized water was used for basal medium. Reagents and samples were kept at laboratory temperature of 29°C.

Table 4. Effect of heating temperature on color-development.

Temperature of water bath, °C	Time of heating, min. 15	25	35	45	60	2	5	10
	Corrected absorbance for 0.4 ppm NH_3–N*					Temperature in tube, °C		
60	.377	.389	.398	.398	.408	53	59	60
70	.400	.406	.410	.410	.409	66	69	70
75	.408	.408	.409	.409	.409	68	74	75
80	.409	.404	.402	.400	.400	69	78	80
90	.389	.382	.370	.365	.367	79	87	89

* Corrected for reagent blank (0.009–0.014 Abs.).
Deionized water was used for basal medium.

pH of sample To know the effect of pH of sample, both deionized water and N/50 sodium acetate containing either hydrochloric acid or sodium hydroxide were used for the basal medium. As shown in Figure 1 the sensitivity did not change more than 2.5 percent from the sample of deionized water in the range between pH's 2.6 and 7.2 measured after addition of reagent F from which acetate buffer was omitted. As the buffer capacity, in general, varied among samples of same pH value, the pH measured after addition of reagent F was thought more significant. So the addition of acetate buffer

to phenol was undertaken to increase the buffer capacity. From the titration curve of reagent F and from the result shown in Figure 1, the present method is thought applicable to the sample whose alkalinity at pH 4.3 is less than 10 meq/*l* or whose acidity at pH 4.3 is less than 1 meq/*l*, if the alkalinity (or acidity) at pH 4.3 nearly equals to that at pH 8.0.

pH of the colored solution Immediately after adding reagent G, very small volume of either 9 N surfuric acid or 60 percent potassium hydroxide was mixed. As shown in Figure 2 the optimal pH's of colored solution were 11.1 for deionized water and 10.8 for sea water samples, where neither acid nor base was added. This fact also supports employing the composition of the reagents as used in the present method.

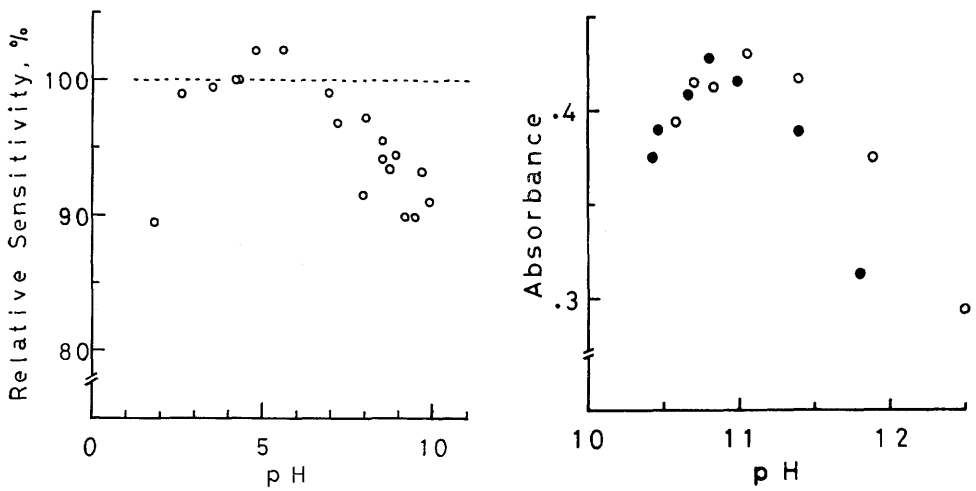

Fig. 1. Effect of pH of solutions after the addition of phenate mixture (F). The abcissa is pH of solutions after the addition of phenate mixture (F). The ordinate is the relative sensitivity, the ratio of absorbance of samples to that of deionized water ones.

Fig. 2. Effect of pH of the colored solution. The abcissa is the pH of colored solutions. The pH was adjusted by mixing very small volume (less than 0.15 m*l*) of either sulfuric acid or potassium hydroxide immediately after adding reagent G. The ordinate is the corrected absorbance of samples containing 0.4 ppm NH_3–N. Open circles are of deionized water samples and solid ones are of sea water samples.

Chlorinity of sample Samples of various chlorinity were made by diluting ammonia-free sea water with deionized water, and the effect of chlorinity was examined as shown in Table 5. With the allowance of 5 percent to relative error or of 5 ppb N to blank, the present method may be applicable to the sample of chlorinity below 28‰.

Though it is rather easy to estimate the recovery of ammonia with a given method, it is very difficult to obtain the blank value of complex mixture such as sea water. It is desirable to prepare ammonia-free artificial sea water, but the chemicals necessary to prepare are usually suspected of contamination with ammonia. SAGI[5] adopted *a priori* that the blank absorbance of sea water was same to that of distilled water, but it is not

Table 5. Salt effect.

Chlorinity, ‰	Absorbance at the concentrations of 0.4 ppm N	0 ppm N	100 Abs. for 0.4 ppm N	Error in estimation** at the concentrations of 0.4 ppm N, %	0 ppm N, ppb N
0	48.68* (1.6)	1.29* (0.3)	47.39		
0.61	50.23 (0.4)	0.93 (0.1)	49.30	3.27	−3.1
3.10	49.90 (0.3)	1.33 (0.4)	48.58	2.59	0.32
6.20	49.35 (0.7)	1.65 (0.9)	47.70	1.42	3.1
9.30	48.98 (0.5)	1.53 (0.7)	47.45	0.63	2.0
12.40	48.68 (0.8)	1.50 (0.5)	47.18	0	1.8
15.50	48.55 (0.4)	1.35 (0.3)	47.20	−0.26	0.5
18.59	48.00 (0.3)	1.78 (0.1)	46.23	−1.42	4.1
21.69	47.73 (0.7)	1.40 (0.3)	46.33	−2.00	0.9
24.79	47.20 (0.2)	1.75 (0.3)	45.45	−3.11	3.9
27.89	47.05 (0.4)	1.78 (0.1)	45.28	−3.43	4.1
30.99	45.53 (0.5)	2.00 (0.4)	43.53	−6.65	6.0

* Mean (range) of four determinations ($\times 10^{-2}$Abs.).

** Estimation was made by using the parameters of calibration with deionized water.

always true as described by MANABE[10]. TETLOW-WILSON[19] reasonably employed the preliminary reaction between phenate and hypochlorite reagents before addition of sample to obtain the reagent blank: since no color formation was usually observed when hypochlorite was added first, the only ammonia in phenate reagent was thought to contribute to the color formation. But using freshly prepared deionized water for the blank, the blank absorbance of ordinary sequence of addition was 0.0140 with the present method, while that of phenate-hypochlorite-deionized water sequence was 0.0030. This difference corresponded to 9.3 ppb of ammoniacal nitrogen, since the sensitivity was about 850 ppb N/Abs. The specific conductance of the deionized water was less than 2×10^{-7} mho/cm which corresponded to 1–100 n mole/*l* order of mono-valent electrolyte solution[20,21]. So it is reasonable to explain the difference between the blank absorbances rather by the effect of different ionic concentration of reaction mixtures than by the contamination of deionized water with ammonia. Another test also supported this explanation: using phenate mixture (F) which was intentionally contaminated with ammonia, the blank absorbance of ordinary sequence of addition was larger than that of phenate-hypochlorite-deionized water sequence of addition. Therefore the procedure of TETLOW-WILSON[19] can not be applied to estimate the blank absorbance without another reason. But in the present method the reagent blank did not vary with the chlorinity of samples as shown in Table 5.

Interferences The effect of several other substances which were dissolved in N/25 sodium acetate solution was tested at two concentrations of ammonia, *i.e.*, 0 and 0.4 ppm N. The analyses were made in triplicate and the calibration was made with acetate solution in each run. The results are summarized in Tables 6 and 7. The limit of error in recovery at the probability of 0.95 was estimated about 5 percent from the pooled standard

deviation within batch, and that in blank was about 6 ppb N. Though not shown in the Tables, 0.1 percent addition of acetone, toluence or xylene did not interfer, but that of either thymol or chloroform did. Hydrogen peroxide at the concentration of 19 ppm reduced the recovery to 38 percent.

Calibration Using deionized and ammonia-free sea water for basal medium, four determinations were made at each concentration. The results are given in Table 8.

Table 6. Influence of co-existing substances.

Substances		Recovery of ammonia, %			Blank as ammonia, ppb N		
		.25	5	50	.25	5	50 ppm
Al	$Al_2K_2(SO_4)_4$	101	104	103	−11	−16	−4
Ba	$BaCl_2$	101	102	100	−4	−1	3
Cu	$CuSO_4$	96	—	—	3	—	—
Fe	$FeSO_4$	97	99	93	15	7	13
	$FeCl_3$	95	96	99	20	15	24
Hg	$HgCl_2$	101	85	70	−9	−4	3
Pb	Pb acetate	98	102	99	−2	3	7
Zn	$ZnSO_4$	99	101	98	6	−2	10
B	H_3BO_3	101	100	99	1	1	−1
Br	KBr	100	97	99	0	4	5
I	KI	100	101	99	0	−6	0
P	Na_2HPO_4		104	100		−5	−4
CrO_4	K_2CrO_4	99	98	100	−5	−8	−1
Cr_2O_7	$K_2Cr_2O_7$	99	101	100	8	−12	3
MnO_4	$KMnO_4$	101	100	81	−4	1	−17
SO_3	Na_2SO_3	105	102	101	−10	−8	−11
S_2O_3	$Na_2S_2O_3$	97	95	61	−5	−10	−18
S	Na_2S	96	93	8	9	56	67
	〃 +Zn*	97	95	84	27	27	11
L-ascorbic acid		102	101	95	1	−11	−15
Oxalic acid		95	100	100	8	−5	6
Glucose		100	98	95	−2	−1	−9
Saccharose		101	101	101	1	2	2
N	$NaNO_2$	99	96	89	1	11	14
	KNO_3	98	97	97	4	13	12
	KCN	102	96	79	2	15	111
	KSCN	102	100	13	0	16	−5
	$(NH_2)_2H_2SO_4$	100	91	16	10	51	295
	$(NH_2OH)_2H_2SO_4$	97	—	—	57	—	—
	Urea	103	102	102	−1	−1	5
	Thiourea	101	90		−14	1	
	CH_3NH_2HCl	100	74	—	24	315	—
	$(CH_3)_2NHHCl$	96	42	7	7	5	32
	$(CH_3)_3NHCl$	94	25	—	11	−9	14
	Creatine	103	91		−12	−16	
	Creatinine	98	85		−12	−16	
	Uric acid	99	97		−11	5	

Basal medium was N/25 sodium acetate solution (0 and 0.4 ppm NH_3-N).

* Supernatant after addition of zinc acetate was used as sample.

Table 7. Influence of L-amino acids.

L-amino acids	.25	5	.25	5 ppm N
	Recovery of ammonia, %		Blank as ammonia, ppb N	
Alanine	97	71	2	87
Arginine	100	100	3	4
Aspartic acid	97	57	4	−9
Cysteine	96	18	−7	−2
Cystine	93	5	2	−19
Glutamic acid	96	48	2	68
Glycine	96	21	−7	2
Histidine	102	85	−5	1
Hydroxyproline	99	62	−11	−17
Isoleucine	100	14	−8	−29
Leucine	99	77	3	36
Lysine	97	75	9	58
Methionine	101	34	−11	−16
Ornitine	100	63	−3	17
Phenylalanine	100	49	3	3
Proline	95	20	−2	−7
Serine	90	23	8	30
Threonine	96	22	1	−32
Tryptophan	100	96	−7	10
Tyrosine	100	60	−6	9
Valine	96	10	2	−33

Basal medium was N/25 sodium acetate solution (0 and 0.4 ppm NH_3–N).

Table 8. Sensitivity and precision of ammonia determinations.

Ammonia-N, ppm	Deionized water				Sea water, 14.13‰ Cl			
	100 Abs. mean	R_4*	Δ Abs. per ppm N	E** ppb N	100 Abs. mean	R_4*	Δ Abs. per ppm N	E** ppb N
0	1.4	0			1.53	0.3		1
0.1	12.65	1.0	1.125	−4	13.43	0.1	1.190	3
0.2	24.65	0.4	1.163	−2	25.55	0.3	1.201	6
0.4	48.38	0.1	1.174	−1	47.85	0.4	1.158	−4
0.6	71.75	0.1	1.173		71.00	0.5	1.158	−6
0.8	94.28	1.1	1.161	−8	90.53	0.4	1.113	−40
1.0	116.93	1.1	1.155	−15	112.70	1.4	1.112	−51
1.2	138.23	0.2	1.140	−34	133.54	0.3	1.100	−73
1.4	160.55	0.7	1.137	−42	150.03	1.2	1.061	−132

* Range of four determinations.

** Error in estimation by using parameters at the concentrations of 0 and 0.6 ppm NH_3–N in deionized water.

BEER's law was applicable up to a concentration even above 1.0 ppm of ammoniacal nitrogen in deionized water samples, but in sea water samples it was applicable only to a concentration of 0.6 ppm N. Employing the absorbances at the concentrations of 0 and 0.6 ppm N in deionized water for the blank and standard, errors in estimation were

calculated and shown in Table 8. The standard deviation within batch was almost same at each concentration. The standard deviation of each determination was 0.0026 Abs. or about 2.2 ppb of ammoniacal nitrogen, computed from pooled ranges of four determinations within batch. The limit of error at the probability of 0.99 was 4.8 ppb N or 0.0056 Abs. for the difference between means of each concentration, though neglecting the variance between batches. By allowing the larger of this limit and of the relative error of 1 percent, the present method may be applicable up to a concentration of 0.6 ppm of ammoniacal nitrogen.

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