Disappearance of Docosahexaenoic and Eicosapentaenoic Acids from Cultures of Mixed Ruminal Microorganisms

A. A. AbuGhazaleh and T. C. Jenkins

Department of Animal and Veterinary Sciences,
Clemson University, Clemson, SC 29634

ABSTRACT

Previous studies showed conflicting results regarding the ability of ruminal microorganisms to hydrogenate docosahexaenoic acid (C22:6, DHA) and eicosapentaenoic acid (C20:5, EPA). To determine the disappearance of DHA and EPA from mixed ruminal cultures, 2 ruminal in vitro experiments were conducted using graded levels of DHA and EPA. The first experiment examined DHA added at 0, 5, 10, 15, and 20 mg per culture flask. In the second experiment, EPA was added at 0, 5, 10, and 15 mg per culture flask. Docosahexaenoic acid and EPA were incubated in triplicate in 125-mL flasks, and 5 mL of culture contents was taken at 0, 12, and 24 h for fatty acid analysis by gas liquid chromatography. After 24 h of incubation, 4.1, 4.1, 4.0, and 3.3 mg of DHA disappeared from the 5, 10, 15, and 20 mg of DHA cultures, respectively. In the second experiment, 5, 8.3, and 7.1 mg of EPA disappeared after 24 h of incubation for the 5-, 10-, and 15-mg EPA cultures, respectively. Addition of DHA to cultures increased *trans*-C18:1 fatty acid accumulation by 105, 91, 82, and 74% for the 5, 10-, 15-, and 20-mg cultures, respectively, compared with control. The addition of EPA increased *trans*-C18:1 fatty acid accumulation by 56, 64, and 55% for the 5-, 10-, and 15-mg EPA cultures, respectively, compared with control. Addition of DHA and EPA to cultures caused a reduction in C18:1 n-9 and C18:2 n-6 biohydrogenation compared with control. Results from these experiments clearly demonstrate the ability of ruminal microorganism to transform DHA and EPA to other fatty acids causing their disappearance from cultures. (**Key words:** docosahexaenoic acid, eicosapentaenoic acid, biohydrogenation)

Abbreviation key: BH = biohydrogenation, DHA = docosahexaenoic acid, EPA = eicosapentaenoic acid, FA = fatty acid.

INTRODUCTION

There is increasing information about the nutritional and health benefits of the long chain omega-3 polyunsaturated fatty acids (FA), docosahexaenoic acid (C22:6, DHA) and eicosapentaenoic acid (C20:5, EPA) for humans (Simopoulos, 1998). Major health benefits ascribed to omega-3 FA include reduced risk of cardiovascular disease, diabetes, hypertension, arthritis, and cancer (Leaf and Weber, 1988; Leaf and Korng, 1998; Sheard, 1998). There is also some evidence that the pathophysiology of major depression is associated with a deficiency of omega-3 FA (Maes et al., 1999).

Marine oils derived from fish or algae are the major source of omega-3 FA in animal diets. Different attempts were made to increase the concentration of DHA and EPA in milk fat by adding fish oil and algae to cow diets (Franklin et al., 1999; Offer et al., 1999; Chilliard et al., 2000; Donovan et al., 2000). However, the apparent efficiency of transfer of these FA from diets to milk was very low (<20%; Franklin et al., 1999; Donovan et al., 2000). These low transfer efficiencies for DHA and EPA were attributed to their association with plasma lipoproteins, which are not good substrates for mammary lipoprotein lipase (Mansbridge and Blake, 1997), or to their preferential partition towards other tissues in the body (Ashes et al., 1992). However, Doreau and Chilliard (1997) attributed their low transfer efficiencies to extensive biohydrogenation (BH) in the rumen. In contrast to Doreau and Chilliard findings, Ashes et al. (1992) reported negligible BH for DHA and EPA when fish oil was incubated with ruminal contents in vitro for 24 h under anaerobic conditions. Therefore, ruminal BH of DHA and EPA remains a subject of debate.

A previous study by Gulati et al. (1999) reported a reduction in BH for DHA and EPA when fish oil was incubated at a level higher than 1 mg/mL of ruminal fluid using batch cultures. Therefore, our studies were initiated to examine the disappearance of DHA and EPA from ruminal cultures when these FA were incubated at different levels. We are not aware of any published study that used a pure form of free DHA or EPA to examine their disappearance from mixed ruminal cultures.

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Corresponding author: A. A. AbuGhazaleh; e-mail: aabugha@clemson.edu.

MATERIALS AND METHODS

Docosahexaenoic acid and EPA were purchased from Sigma-Aldrich Chemical Company (>99% purity; St. Louis, MO), dissolved in ethanol, and stored at -5°C until used. Ruminal contents were obtained from a fistulated Holstein cow and strained through 2 layers of cheesecloth and transported to the laboratory in a sealed container and used within 20 min. Treatments for the first experiment were control (no DHA) or control with 5, 10, 15, or 20 mg of added DHA. Treatments in the second experiment were control (no EPA) or control with 5, 10, or 15 mg added EPA. Assuming DHA and EPA each approximately represent 10% fish oil FA, the 5, 10, 15, and 20 mg of added FA would be equivalent to incubating fish oil at 1, 2, 3, and 4 mg/mL of ruminal fluid. Docosahexaenoic acid and EPA in ethanol (400 μL) were added directly into each culture. Cultures were maintained in 125-mL Erlenmeyer flasks containing 500 mg of finely ground TMR, 10 mL of the strained ruminal fluid, 40 mL of media, and 2 mL of reducing solution according to Goering and VanSoest (1970). The TMR used in cultures was a 50:50 blend of forage (corn silage) and concentrate (corn, soybean meal minerals, and vitamins). Cultures were run in triplicate at 39°C under anaerobic conditions. To determine the initial and final DHA and EPA concentrations, 5-mL samples were taken from each culture flask at 0, 12, and 24 h while being stirred with a magnetic bar under a stream of CO_2 , placed immediately in an ice bath, and then stored at -5°C .

Samples were freeze dried and then methylated according to Kramer et al. (1997) and analyzed for FA by GLC. Methylated FA were separated using a fused silica capillary column (100 m $\times$ 0.25 mm, i.d. $\times$ 0.20 μm thickness), CP-SiL88 (Chrompack, Ravitan, NJ). The split ratio in the injector port (160°C) was 100:1 with a column flow of 1.5 mL/min of He. Oven temperature was programmed for 160°C for 4 min, then increased from 160 to 215°C at $4^{\circ}\text{C}/\text{min}$, and finally held at 215°C for 21 min. The injector and detector temperatures were 250°C . Heptadecanoic acid (C17:0) was added to all samples as an internal standard. Data were analyzed by ANOVA using the general linear model procedure of SAS (SAS Inst., Inc., Gary, NC). Orthogonal contrast statements were used to compare treatments effect at each sampling time (0, 12, and 24 h). Planned comparisons were: 1) control versus DHA and EPA, 2) linear effect, and 3) quadratic effect. Cubic effect was tested and found not significant. Significance was determined at $P < 0.10$.

RESULTS

The changes in mg of FA in ruminal cultures with the addition of DHA and EPA are presented in Tables

1 and 2. After 12 h of incubation, disappearance of DHA from cultures showed a linear effect ($P < 0.10$, Table 1). Disappearance was highest with the 5-mg DHA level averaging 3.0 mg/culture, intermediate with 10- and 15- mg of DHA levels (2.5 and 2.2 mg), and least with the 20-mg DHA level (1.4 mg; Table 1). The disappearance of DHA from cultures was further increased after 24 h of incubation for all levels, averaging 4.1, 4.1, 4.0, and 3.3 mg per culture for the 5-, 10-, 15-, and 20-mg DHA levels, respectively (Table 1). Total DHA disappeared over 24 h was similar for the 5-, 10-, and 15-mg DHA levels, and they were all higher than the 20-mg DHA level. As for EPA disappearance, 4.3, 5.4, and 4.3 mg of EPA disappeared after 12 h of incubation for the 5-, 10-, and 15-mg EPA levels, respectively, resulting in quadratic effect ($P < 0.10$; Table 2). Disappearance of EPA increased as incubation time increased from 12 to 24 h, resulting in the 5-mg cultures being cleared of EPA and the majority of the EPA being lost in the 10- and 15-mg cultures (8.2 and 7.1 mg disappeared, respectively). The disappearance of EPA from cultures over time was much higher than that of DHA (Figure 1). After 24 h of incubation, some unidentified long-chain FA were detected around DHA and EPA peaks in cultures (Figure 2; Tables 1 and 2).

The addition of DHA and EPA to cultures at different levels affected the BH of other FA (Tables 1 and 2). After 24 h of incubation, *trans*-C18:1 FA concentrations were higher ($P < 0.10$) with added DHA and EPA compared with control. Concentrations of *trans*-C18:1 FA were highest for the 5-mg DHA level and there was a linear ($P = 0.06$) decrease for *trans*-C18:1 FA concentration with increasing DHA level (Table 1). Unlike control cultures where the concentration of C18:1 n-9 decreased after the 24 h of incubation as a result of BH, addition of DHA increased ($P < 0.10$) the concentration of C18:1 n-9, while the addition of EPA caused no changes in the concentration of C18:1 n-9 in cultures after the 24 h of incubation. Addition of DHA and EPA to cultures also resulted in no changes in the concentration of C18:0 after 24 h of incubation compared with control, where the concentration of C18:0 increased by approximately 150% as a result of complete BH of unsaturated FA (Tables 1 and 2). After 24 h of incubation, the concentration of short-chain FA ($<\text{C16:0}$) in ruminal cultures were linearly decreased ($P < 0.10$) as the levels of DHA and EPA in cultures increased (Tables 1 and 2).

DISCUSSION

The principal objective of these studies was to assess the disappearance of DHA and EPA from ruminal cultures when incubated at different levels. Disappearance of DHA and EPA from cultures can be caused

Table 1. Changes in milligrams of fatty acids per culture flask over time with added docosahexaenoic acid (DHA) (mg/flask).

Fatty acids		Treatment						Contrast ¹	
		Control	5mg DHA	10mg DHA	15mg DHA	20mg DHA	SE	Lin	Quad
<C16:0									
	0-h	14.5	14.7	14.1	13.7	13.7	0.53	0.20	0.58
	12-h	21.0	20.3	19.4	18.4	18.6	0.30*	0.03	0.17
	24-4	25.4	24.3	23.3	22.9	23.0	0.27*	0.03	0.08
C18:0									
	0-h	10.3	10.1	10.2	10.1	10.0	0.13	0.21	0.66
	12-h	20.6	10.4	10.3	10.2	10.3	0.06*	0.21	0.10
	24-4	25.8	11.0	10.3	10.2	10.1	0.07*	0.01	0.01
C18:1 <i>cis</i> -9									
	0-h	8.1	7.9	7.5	7.8	7.1	0.33	0.26	0.91
	12-h	5.6	7.8	7.9	9.2	8.1	0.13*	0.08	0.05
	24-4	4.4	7.3	8.4	8.9	7.9	0.41*	0.21	0.07
C18:1 <i>trans</i>									
	0-h	1.6	1.6	1.5	1.7	1.4	0.09	0.17	0.70
	12-h	6.8	11.0	9.4	9.2	7.9	0.36*	0.03	0.72
	24-4	7.2	15.3	14.1	13.5	12.3	0.53*	0.06	0.65
C18:2									
	0-h	13.7	13.5	12.8	12.4	12.3	0.38*	0.10	0.36
	12-h	4.8	5.9	7.6	7.3	8.4	0.45*	0.07	0.58
	24-4	1.9	2.2	3.1	3.2	3.7	0.29*	0.04	0.42
C18:3									
	0-h	0.7	0.6	0.7	0.6	0.6	0.01	0.09	0.50
	12-h	0.2	0.2	0.2	0.3	0.3	0.05	0.54	0.50
	24-4	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
C22:0									
	0-h	0.1	0.1	0.1	0.1	0.1	>0.01	0.74	0.99
	12-h	0.2	0.2	0.1	0.1	0.2	>0.01*	0.27	0.03
	24-4	0.2	0.2	0.1	0.1	0.1	>0.01*	0.40	0.51
C24:0									
	0-h	0.1	0.1	0.1	0.1	0.1	>0.01	0.27	0.23
	12-h	0.2	0.2	0.2	0.2	0.2	>0.01	0.62	0.52
	24-4	0.2	0.2	0.2	0.2	0.2	0.02	0.07	0.09
C22:6 (DHA)									
	0-h	0.0	5.2	10.2	14.6	18.6	0.09*	<0.01	0.03
	12-h	0.0	2.2	7.8	12.4	17.2	0.05*	<0.01	0.01
	24-4	0.0	1.2	6.1	10.6	15.3	0.28*	<0.01	0.66
Others ²									
	0-h	0.0	0.1	0.0	0.1	0.1	0.03	0.71	0.17
	12-h	0.3	1.2	0.5	0.3	0.3	0.03*	<0.01	<0.01
	24-4	0.3	2.2	1.0	0.9	0.9	0.20*	0.04	0.09
Total FA/flask									
	0-h	48.3	54.5	57.5	61.0	64.1	2.27*	0.06	0.85
	24-h	64.8	64.4	66.9	70.8	73.9	1.17*	0.02	0.49
DHA disap ³									
	12-h	0.0	3.0	2.5	2.2	1.4	0.05	<0.01	0.12
	24-4	0.0	4.0	4.1	4.0	3.3	0.15	0.07	0.11

¹Contrast = probability of a linear (Lin) or quadratic (Quad) effect of the level of added DHA.²Unidentified long-chain fatty acids.³DHA disappearance (mg/flask).*Treatment effect (control vs. DHA), $P < 0.10$.

by: 1) their transformation into other C22:6 and C20:5 isomers as a result of enzymatic isomerization, 2) hydrogenation of their double bonds, and 3) shortening of their carbon chain. The disappearance of DHA and EPA from cultures from cultures after 24 h of incubation clearly demonstrates the ability of ruminal microorganisms to transform DHA and EPA into other FA. Although both DHA and EPA disappeared from cultures after 24 h of incubation, the amount that disappeared

was not equal. The disappearance of EPA was greater (Figure 1) than that of DHA when incubated at similar levels (5, 10, and 15 mg per culture). Increasing the levels of DHA and EPA in cultures seems to have some influence on their disappearance. For example, the total amount of that DHA disappeared from cultures decreased from 4.1 to 3.3 mg per culture when the DHA level increased from 15 to 20 mg, respectively. The same trend was also seen when EPA level increased from 10

Table 2. Changes in milligrams of fatty acids per culture flask over time with added eicosapentaenoic acid (EPA) (mg/flask).

Fatty acids		Treatment				SE	Contrast ¹	
		Control	5mg EPA	10mg EPA	15mg EPA		Lin	Quad
C16:0	0-h	15.9	15.9	15.5	15.8	0.25	0.86	0.36
	12-h	21.9	22.1	20.4	20.5	0.98	0.32	0.44
	24-4	27.7	27.7	26.1	25.3	0.23*	0.02	0.28
C18:0	0-h	11.7	11.3	11.2	11.3	0.15*	0.83	0.49
	12-h	18.7	12.6	11.1	11.4	0.48*	0.19	0.20
	24-4	26.7	19.5	11.8	11.8	0.06*	<0.01	<0.01
C18:1 <i>cis</i> -9	0-h	8.6	9.6	9.0	9.1	0.14	0.05	0.33
	12-h	6.4	8.0	8.8	9.4	0.40*	0.10	0.88
	24-4	4.6	5.7	9.3	9.5	0.34*	0.01	0.08
C18:1 <i>trans</i>	0-h	2.0	1.8	2.1	1.7	0.22	0.65	0.32
	12-h	7.4	11.3	10.2	9.7	0.27*	0.04	0.40
	24-4	8.8	13.7	14.4	13.4	0.43*	0.02	0.28
C18:2	0-h	14.9	14.8	14.1	14.5	0.42	0.82	0.39
	12-h	7.0	7.7	8.4	10.0	0.6*	0.09	0.48
	24-4	2.9	3.0	4.9	6.2	0.32*	0.02	0.64
C18:3	0-h	0.9	0.9	0.9	0.9	0.02	0.93	0.94
	12-h	0.4	0.4	0.4	0.4	0.02	0.68	0.65
	24-4	0.2	0.2	0.2	0.2	0.01	0.55	0.72
C22:0	0-h	0.2	0.2	0.2	0.2	0.01	0.68	0.81
	12-h	0.2	0.2	0.2	0.2	0.00	0.58	0.78
	24-4	0.2	0.2	0.2	0.2	0.01	0.30	0.51
C24:0	0-h	0.2	0.2	0.2	0.2	0.01	0.91	0.89
	12-h	0.2	0.2	0.2	0.2	0.01	0.68	0.65
	24-4	0.2	0.2	0.2	0.2	0.02	0.32	0.36
C20:5 (EPA)	0-h	0.0	4.6	9.7	14.8	0.19*	<0.01	0.76
	12-h	0.0	0.4	4.3	10.2	0.29*	<0.01	0.08
	24-4	0.0	0.0	1.6	7.6	0.21*	<0.01	0.01
Others ²	0-h	0.0	0.0	0.1	0.1	0.02*	0.03	0.57
	12-h	0.5	2.1	3.3	3.1	0.13*	0.03	0.03
	24-4	0.5	2.8	5.8	4.1	0.17*	0.02	0.03
Total FA (flask)	0-h	55.3	59.6	63.5	69.1	1.47*	0.04	0.69
	24-4	72.5	73.7	74.8	79.9	0.29*	>0.01	>0.01
EPA disap ³	12-h	0.0	4.2	5.4	4.5	0.18	0.26	0.02
	24-4	0.0	4.7	8.2	7.2	0.05	<0.01	<0.01

¹Contrast = probability of a linear (Lin) or quadratic (Quad) effect of the level of added EPA.²Unidentified long-chain fatty acids.³EPA disappearance (mg/flask).*Treatment effect (control vs. EPA), $P < 0.10$.

to 15 mg. Gulati et al. (1999) also observed the same effect when they incubated fish oil (source of DHA and EPA) at different levels in cultures of ruminal microorganisms. They observed a higher degree of BH for DHA and EPA when fish oil was incubated at less than 1 mg/mL of ruminal fluid, but as the level of fish oil increased in cultures, the degree of BH for DHA and EPA decreased. The contrasting results regarding DHA and EPA BH between Ashes et al. (1992), who reported

negligible hydrogenation for DHA and EPA, and that of Doreau and Chilliard (1997), who reported >80% BH for DHA and EPA, may be explained by the level of fish oil used in these experiments. For example, Ashes et al (1992) incubated fish oil at 5 mg/mL of ruminal fluid, which would be equivalent to incubating 50 mg of DHA and EPA in our studies.

When comparing the disappearance of DHA and EPA with free C18:2 n-6 in mixed ruminal cultures (Figure

Figure 1. Disappearance of docosahexaenoic acid, eicosapentaenoic acid, and C18:2 n-6 from cultures after 24 h of incubation. * Adapted from Jenkins (unpublished, 2001).

1), C18:2 n-6 disappearance increased as the level in cultures increased, reaching a maximum at 25 mg, then leveling off demonstrating a greater capability of ruminal microorganisms to hydrogenate C18:2 n-6. These differences in disappearance of DHA, EPA, and C18:2 n-6 from cultures when incubated at similar levels may suggest either the presence of different isomerase enzymes with different activities for each FA or the same isomerase enzyme but with different affinity to FA.

The additions of DHA and EPA to cultures have also influenced the disappearance of C18:2 n-6 (Tables 1 and 2). Although C18:2 n-6 was higher ($P < 0.10$) at 0 h for control compared with other DHA levels, C18:2 n-6 concentration after 24 h of incubation was lower ($P < 0.10$) for the control indicating a reduction in C18:2 n-6 BH with added DHA. The BH of C18:2 n-6 linearly ($P < 0.10$) decreased as the level of DHA in cultures increased (Table 1). Compared with control, BH of C18:2 n-6 was reduced by 20% when 20 mg of DHA was added to cultures (Table 1). The same effect was seen when EPA was added (Table 2). The addition of 10 and 15 mg of EPA to cultures reduced the disappearance of C18:2 n-6 by 20 and 30%, respectively, compared with control. This reduction in C18:2 n-6 BH may explain the increase in milk C18:2 n-6 concentrations observed by Cant et al. (1997), Offer et al. (1999), and Kitessa et al. (2001b) when fish oil was added to animals diet.

The disappearance of oleic acid (C18:1 n-9) from cultures showed a different pattern than that of C18:2 n-6. (Tables 1 and 2). The addition of 5 mg DHA to cultures reduced the disappearance of C18:1 n-9 from 46% (control) to 10%. Furthermore, the incubation of DHA at 10, 15, and 20 mg levels unexpectedly increased the concentration of C18:1 n-9 in cultures after 24 h of

incubation (Table 1). A similar decline in the disappearance of C18:1 n-9 was also seen with the addition of 10 and 15 mg EPA to cultures (Table 2). This decline in C18:1 n-9 disappearance with added DHA and EPA may have resulted from inhibiting the reductase enzyme activity in ruminal microorganisms, which is responsible for the terminal hydrogenation of C18:1 n-9 to C18:0. The possibility of DHA and EPA or their derivatives inhibiting reductase activity is supported by the fact that C18:0 levels did not increase after 24 h of incubation when DHA and EPA were added to cultures compared with control (Tables 1 and 2). An alternative explanation for the decline in C18:1 n-9 disappearance is that DHA and EPA may have promoted the conversion of *trans* FA (such as *trans*-9 C18:1) into C18:1 n-9. Proell et al. (2002) reported a small conversion of C18:1 *trans*-9 into C18:1 n-9 when they used ^{13}C -labeled *trans*-9 C18:1 in vitro. Even though the possibility of C18:1 n-9 being synthesized directly from DHA and EPA is unlikely due to the absence of the double bond at carbon 9 in DHA and EPA, it is still a possibility that a migration of *cis*-double bond could have occurred resulting in C18:1 n-9 formation. Others have also reported a reduction in C18:0 concentration in ruminal digesta (AbuGhazaleh et al., 2002), duodenal FA flow (Scollan et al., 2001) and in milk fat content (Offer et al., 1999; Donovan et al., 2000) when fish oil was added to animals diet.

Additions of DHA and EPA to ruminal cultures have also affected *trans*-C18:1 FA concentrations (Tables 1 and 2). The concentration of *trans*-C18:1 FA after 24 h of incubation was increased by 105, 91, 82, and 74% when DHA was added at 5, 10, 15, and 20 mg, respectively, compared with control (Table 1). Vaccenic acid (*trans*-11 C18:1) was the major *trans*-C18:1 isomer representing 88, 93, 92, and 95% of total *trans*-C18:1 FA after 24 h of incubation when DHA was added at 5, 10, 15, and 20 mg, respectively (data not shown). A similar increase in *trans*-C18:1 FA concentrations were also seen with added EPA (Table 2). The addition of EPA at 5, 10, and 15 mg increased *trans*-C18:1 FA after 24 h by 56, 64, and 55%, respectively, compared with control. It appears that DHA has a greater ability to promote *trans*-C18:1 FA accumulation compared with EPA. Previous studies have also observed an increase in ruminal (Kitessa et al., 2001a; AbuGhazaleh et al., 2002) and duodenal (Scollan et al., 2001) *trans*-C18:1 FA concentration when fish oil was added to animals diet. This increase in *trans*-C18:1 FA with added DHA and EPA may be caused by inhibiting the reductase activity of ruminal microorganisms, causing the accumulation of *trans*-C18:1 FA in cultures. However, the possibility that DHA and EPA had directly contributed to *trans*-C18:1 FA formation can not be ruled out since

Figure 2. The GLC chromatograms that illustrate the disappearance of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from cultures and their transformation into other fatty acids. A) control at 0 h, B) control at 24 h, C) DHA at 0 h, D) DHA at 24 h, E) EPA at 0 h, F) EPA at 24 h.

the losses in unsaturated FA (C18:1 n-9, C18:2 n-6, C18:3) from cultures after 24 h of incubation did not account for all the increase in *trans*-C18:1 FA with the addition of DHA and EPA (Tables 1 and 2). Additionally, the total sum of all unsaturated C18 FA (C18:1, C18:2 n-6, C18:3) increased in DHA and EPA cultures after 24 h of incubation (Tables 1 and 2).

CONCLUSIONS

The results from these studies demonstrate the ability of microorganisms from the rumen to transform DHA and EPA to other FA. The maximum transformation per culture of ruminal contents was lower for DHA and EPA than for C18:2 n-6. Percentage of DHA and EPA disappeared declines with increasing initial concentrations of FA.

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