Regulation of the biosynthesis of 4,7,10,13,16-docosapentaenoic acid

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It is now established that fatty acid 7,10,13,16-22:4 is metabolized into 4,7,10,13,16-22:5 as follows: 7,10,13,16-22:4 → 9,12,15,18-24:4 → 6,9,12,15,18-24:5 → 4,7,10,13,16-22:5. Neither C4 fatty acid was esterified to 1-acyl-sn-glycero-3-phosphocholine (1-acyl-GPC) by microsomes, whereas the rates of esterification of 4,7,10,13,16-22:5, 7,10,13,16-22:4 and 5,8,11,14-20:4 were respectively 135, 18 and 160 nmol/min per mg of microsomal protein. About four times as much acid-soluble radioactivity was produced when peroxisomes were incubated with [3-14C]9,12,15,18-24:4 compared with 6,9,12,15,18-24:5. Only [1-14C]7,10,13,16-22:4 accumulated when [3-14C]9,12,15,18-24:4 was the substrate, but both 4,7,10,13,16-22:5 and 2-trans-4,7,10,13,16-22:6 were produced from [3-14C]6,9,12,15,18-24:5. When the two C4 fatty acids were incubated with peroxisomes, microsomes and 1-acyl-GPC there was a decrease in the production of acid-soluble radioactivity from [3-14C]6,9,12,15,18-24:5, but not from [3-14C]9,12,15,18-24:4. The preferential fate of [1-14C]4,7,10,13,16-22:5, when it was produced, was to move out of peroxisomes for esterification into the acceptor, whereas only small amounts of 7,10,13,16-22:4 were esterified. By using 3H-labelled 9,12,15,18-24:4 it was shown that, when 7,10,13,16-22:4 was produced, its primary metabolic fate was degradation to yield esterified arachidonate. Collectively, the results show that an inverse relationship exists between rates of peroxisomal β-oxidation and of esterification into 1-acyl-GPC by microsomes. Most importantly, when a fatty acid is produced with its first double bond at position 4, it preferentially moves out of peroxisomes for esterification to 1-acyl-GPC by microsomes, rather than being degraded further via a cycle of β-oxidation that requires NADPH-dependent 2,4-dienoyl-CoA reductase.

INTRODUCTION

Several studies have shown that microsomes do not contain an acyl-CoA-dependent 4-desaturase [1–6]. When the fatty acids 7,10,13,16-22:4 and 7,10,13,16,19-22:5 are produced in the endoplasmic reticulum, they are chain-elongated to 9,12,15,18-24:4 and 9,12,15,18,21-24:5 respectively. These two acids are subsequently desaturated at position 6 to yield 6,9,12,15,18-24:4 and 6,9,12,15,18,21-24:6 [1,2]. These endoplasmic reticulum-derived metabolites must then move to a site, most probably the peroxisomes, for partial β-oxidation [5,7]. The chain-shortened metabolites formed by one or more cycles of β-oxidation are not esterified into 1-acyl-sn-glycero-3-phosphocholine (1-acyl-GPC) by peroxisomes [8], but rather they must move back to the endoplasmic reticulum, where they are used for phospholipid biosynthesis [8,9].

Prior to the discovery that microsomes do not have a 4-desaturase, the biosynthesis of polyunsaturated fatty acids and their incorporation into membrane lipids could be viewed as processes that were both localized primarily in the endoplasmic reticulum [10–12]. The revised pathways of unsaturated fatty acid biosynthesis require considerable movement of fatty acids between microsomes and peroxisomes [13]. Any given fatty acid may be used as a substrate for a variety of different reactions. For example, when [3-14C]12,15,18-24:4 was incubated with rat hepatocytes, only small amounts were esterified into phospholipids, which is most probably a microsomal process. In addition, it was possible to detect esterified [1-14C]22:4,n-6 [2]. This finding implies that, when 24:4,n-6 is produced in the endoplasmic reticulum (from 22:4,n-6), some of it must move into the peroxisomes, where it is partially β-oxidized to 22:4,n-6; this then moves back to the endoplasmic reticulum, where it may be used again as a substrate for either esterification or chain elongation. In this type of study it is not possible to determine how much [3-14C]24:4,n-6 is metabolized to 20:4,n-6 via two cycles of β-oxidation, because of loss of the label. In any case, when [3-14C]24:4,n-6 was incubated with hepatocytes, its major measurable fate was desaturation to 24:5,n-6 followed by partial β-oxidation to yield esterified [1-14C]22:5,n-6 [2]. When [3-14C]24:5,n-6 was incubated with hepatocytes, only small amounts were esterified into phospholipids. Its major fate was metabolism to yield esterified [1-14C]22:5,n-6, but again it was not possible to detect product formation after the second cycle of β-oxidation because of loss of label [2].

In the study reported here, unsaturated fatty acids, labelled with 3H at the last two carbon atoms, as well as 14C-labelled acids, were used to determine how peroxisomes and microsomes interact to regulate the synthesis and subsequent esterification of long-chain n-6 fatty acids into membrane lipids using 1-acyl-GPC as an acceptor.

MATERIALS AND METHODS

Materials

ATP, NAD+, NADPH, CoASH, dithiothreitol and essentially fatty acid-free BSA were obtained from Sigma. Lactate dehydrogenase and Nycodenz (Accudenz) were obtained from Boehringer Mannheim and Accurate Chemicals and Scientific Corp. respectively. 1-Palmitoyl-2-sn-[1-14C]glycero-3-phosphocholine was from DuPont NEN. 1-Palmitoyl-2-sn-[6,9,12,15-heneicosatetrenoyl]glycero-3-phosphocholine was syn-

Abbreviation used: 1-acyl-GPC, 1-acyl-sn-glycero-3-phosphocholine. 1 To whom correspondence should be addressed.
the synthesized and purified as described by Paltauf and Hermetter [14]. [19,19,20,20-1H]Ethyl arachidonate [15] was converted into [23,23,24,24-14C]9,12,15,18-tetraenoic acid via a sequence of reactions whereby the ethyl ester of labelled arachidonic acid was reduced to the alcohol with LiAlH₄. The resulting alcohol was converted into the mesylate, which was displaced with cyanide. Following hydrolysis with 5% anhydrous HCl in methanol, the methyl ester of the labelled C₂₀₄₀ was obtained [16]. This sequence of reactions was repeated three more times to yield the desired product. All 1⁻¹⁴C- and 3⁻¹⁴C-labelled acids were made by total organic synthesis [16].

**Isolation of rat liver peroxisomes and microsomes**

Male Sprague–Dawley rats (200–250 g) were fed on Purina chow without or with 0.5% (w/v) clofibrate for 8 days prior to being killed. In essence, the procedure of Das et al. [12], as previously described [17], was used to isolate peroxisomes. Briefly, liver was homogenized in a medium containing 0.25 M sucrose, 1 mM EDTA, 0.1% ethanol and 10 mM Tes, pH 7.5. Nuclei and the heavy mitochondrial fraction obtained by centrifugation at 600 × g and 3300 g respectively were discarded. The light mitochondrial pellet obtained by centrifugation at 25000 × g for 17 min was resuspended in the homogenization buffer and centrifuged at 25000 × g for 15 min. One additional wash was done under the same conditions. The resulting pellet was then resuspended in the homogenization buffer in a volume corresponding to 1 ml for every 2 g of liver. A 2 ml portion of this suspension was then layered on 15 ml of 35% ethanol and 10 mM Tes, pH 7.5. Nuclei and the heavy mitochondrial fraction obtained by centrifugation at 600 × g and 3300 g respectively were discarded. The light mitochondrial pellet obtained by centrifugation at 25000 × g for 17 min was resuspended in the homogenization buffer and centrifuged at 25000 × g for 15 min. One additional wash was done under the same conditions. The resulting pellet was then resuspended in the homogenization buffer in a volume corresponding to 1 ml for every 2 g of liver. A 2 ml portion of this suspension was then layered on 15 ml of 35% (w/v) Nycodenz containing 10 mM Tes, 0.1% ethanol and 1 mM EDTA, and centrifuged at 56800 × g for 45 min. The resulting peroxisomal pellet was suspended in incubation medium, which contained 130 mM KCl and 20 mM Hepes, pH 7.2 [18]. The protein concentration was adjusted to 3 mg/ml, as determined by using the Coomassie Blue reagent (Pierce) with BSA as a standard. In order to prepare microsomes, the supernatant was centrifuged at 110000 × g for 45 min. The microsomal pellet was suspended in 0.6 M KCl/0.1 M phosphate buffer, pH 7.4. After centrifugation at 110000 × g for 45 min, the microsomal pellet was suspended in 130 mM KCl/20 mM Hepes, pH 7.2, and stored at −80 °C.

**Fatty acid activation**

Peroxisomes (10 or 50 µg of protein) were incubated in a total volume of 0.2 ml in a medium that contained 130 mM KCl, 20 mM Hepes, 10 mM MgATP, 0.4 mM CoASH and the sodium salt of the radioactive fatty acid (2 Ci/mmol) bound to BSA in a 2:1 molar ratio (assuming an M₀ of 66000 for BSA). Reactions were initiated by the addition of peroxisomes. The fatty acid concentration varied from 12.5 to 150 µM. After 2 min the reactions were terminated by the addition of 2.5 ml of isopropyl alcohol/heptane/0.05 M H₂SO₄ (40:10:1, by vol.), followed by 1.5 ml of n-heptane and 1 ml of water. The upper layer, containing the unreacted fatty acid, was discarded and the bottom layer was washed twice with 2 ml of heptane [19]. The bottom layers, containing the acyl-CoA's, were transferred to scintillation vials and radioactivity was counted in 10 ml of ACS II (Amersham). Maximum rates of activation were calculated from double-reciprocal plots.

**Microsomal acylation of 1-acyl-GPC**

The CoA derivatives of fatty acids were synthesized via their mixed anhydrides [20]. Maximum rates of acylation were determined spectrophotometrically [21]. Incubations contained 50 µM acyl-CoA, 300 µM 1-acyl-GPC, 2 mM 5,5-dithiobis-(2-nitrobenzoic acid) and 25–200 µg of microsomal protein in 1 ml of 0.1 M Tris/HCl (pH 7.4).

**RESULTS**

Microsomal esterification of fatty acids into 1-acyl-GPC

Our previous studies have shown that arachidonic acid is metabolized via 22:4,6 - 24:4,6 - 24:5,6 - 24:5,7 - 25:2,6.
endoplasmic reticulum [2]. If either of these two C₂₄ acids were used for membrane lipid biosynthesis where they are produced, it would curtail their rapid movement to a site for partial β-oxidation. The results in Table 1 show that neither C₂₄ nor C₂₅ acid was esterified into 1-acyl-GPC by microsomes from rats fed on diets containing acyl-CoA hydrolases [26,27]. It is not known whether the endoplasmic reticulum, they also are acyl-CoAs. Cytosol contains acyl-CoA hydrolases [26,27]. It is not known whether acyl-CoAs can move directly into peroxisomes, or whether they are hydrolysed and subsequently re-activated by the subcellular organelle in which they are to be metabolized. The results in Table 2 show that peroxisomes have the ability to activate C₂₄, C₂₅ and C₂₆ acids, albeit at markedly different rates. As was observed for rates of microsomal acylation, the addition of clofibrate to the diet increased the rates of activation. This effect was somewhat greater for C₂₆ and C₂₅ acids than for the C₂₄ compounds. Microsomes, mitochondria and peroxisomes all contain a long-chain acyl-CoA synthetase [28,29]. Peroxisomes and microsomes, but not mitochondria, contain an enzyme that is designated as a very-long-chain acyl-CoA synthetase [30]. The above studies do not discriminate between these two enzyme activities. Since all of the n-6 acids were activated by peroxisomes, all subsequent studies were carried out by generating acyl-CoAs in situ, which may well be the case in vivo.

Fatty acid activation

Since 24:4, 5n-6 is a poor substrate for acylation into 1-acyl-GPC, the above results imply that, in vivo, it must either be desaturated at position 6 to yield 24:5, 5n-6 or move to peroxisomes for partial β-oxidation to 22:4, 5n-6. The primary metabolic fate of 24:5, 5n-6 must be to move to a site for partial degradation. The true substrates and products for a number of microsomal desaturation and chain- elongation reactions are the acyl-CoAs [23–25]. Presumably when C₂₄ acids are produced in the endoplasmic reticulum, they also are acyl-CoAs. Cytosol contains acyl-CoA hydrolases [26,27]. It is not known whether acyl-CoAs can move directly into peroxisomes, or whether they are hydrolysed and subsequently re-activated by the subcellular organelle in which they are to be metabolized. The results in Table 2 show that peroxisomes have the ability to activate C₂₄, C₂₅ and C₂₆ acids, albeit at markedly different rates. As was observed for rates of microsomal acylation, the addition of clofibrate to the diet increased the rates of activation. This effect was somewhat greater for C₂₆ and C₂₅ acids than for the C₂₄ compounds. Microsomes, mitochondria and peroxisomes all contain a long-chain acyl-CoA synthetase [28,29]. Peroxisomes and microsomes, but not mitochondria, contain an enzyme that is designated as a very-long-chain acyl-CoA synthetase [30]. The above studies do not discriminate between these two enzyme activities. Since all of the n-6 acids were activated by peroxisomes, all subsequent studies were carried out by generating acyl-CoAs in situ, which may well be the case in vivo.

Peroxisomal fatty acid β-oxidation and the esterification of chain-shortened acids into 1-acyl-GPC by microsomes

The results in Figure 1 show that with all four n-6 acids the addition of clofibrate to the diet increased the rate of peroxisomal β-oxidation. When clofibrate was included in the diet, about 30 nmol of acid-soluble radioactivity was produced from [1-14C]7,10,13,16-22:4 at 5 min (Figure 1A), compared with only about 2 nmol when [1-14C]7,10,13,16-22:5 was the substrate (Figure 1B). Similar differences existed for the two C₂₅ acids: after 5 min, about eight times as much acid-soluble radioactivity was produced from [3-14C]9,12,15,18-24:4 as from [3-14C]6,9,12,15,18-24:5 (Figures 1C and 1D respectively).

The results in Figure 1 show that, when peroxisomes from rats fed on a clofibrate-containing diet were incubated with microsomes and 1-acyl-GPC, there was only a small decrease in the production of acid-soluble radioactivity from [1-14C]7,10,13,16-22:4 (Figure 1A) or [3-14C]9,12,15,18-24:4 (Figure 1C). The loss of label from these two fatty acids requires only the enzymes of saturated fatty acid degradation. Conversely, when [1-
Table 3 Incorporation of radioactive fatty acids into phospholipids on incubation of peroxisomes with 1-acyl-GPC and microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Esterified radioactive fatty acid (nmol)</th>
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<tbody>
<tr>
<td>[1-14C]22:4, n=6</td>
<td>15.8 ± 0.3</td>
</tr>
<tr>
<td>[3-14C]24:4, n=6</td>
<td>9.0 ± 1.0</td>
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<tr>
<td>[1-14C]22:5, n=6</td>
<td>–</td>
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<tr>
<td>[3-14C]24:5, n=6</td>
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Characterization of metabolites produced on incubation of [1-14C]-4,7,10,13,16-22:5 and [3-14C]6,9,12,15,18-24:5 with peroxisomes

When [1-14C]22:4, n=6 was incubated alone with peroxisomes, it was only possible to detect unmetabolized substrate when the methyl esters, formed by saponification and subsequent esterification, were analysed by HPLC. When [3-14C]24:4, n=6 was the substrate it was possible to detect both unmetabolized substrate and [1-14C]22:4, n=6. When [1-14C]22:5, n=6 was incubated with peroxisomes, three radioactive metabolites were eluted immediately before unmetabolized substrate (Figure 2A). When [3-14C]24:5, n=6 was incubated under the same conditions, it was possible to detect the same three compounds in addition to [1-14C]22:5, n=6, i.e. compound 4 in Figure 2(B). The UV spectrum of compound 3 was identical with that of authentic methyl-2-trans-4-cis-10:2, which was isolated from the seed oil of the Chinese tallow tree [32]. Compound 3 is thus 2-trans-4,7,10,13,16-22:6, which is the substrate for NADPH-dependent 2,4-dienoyl-CoA reductase, and Δ9,Δ12-dienoyl-CoA isomerase, which is a component of the peroxisomal trifunctional enzyme [31].

The incubations were terminated after 30 min, and methyl esters were prepared and analysed by HPLC. Compounds 1 and 2 are isomers of 3,5,7,10,13,16-22:6, and compounds 3, 4 and 5 are 2-trans-4,7,10,13,16-22:6, 22:5, n=6 and 24:5, n=6 respectively.

Metabolism of 4H-labelled 9,12,15,18-24:4

Since the production of acid-soluble radioactivity from [1-14C]4,7,10,13,16-22:5 (Figure 1B) or [3-14C]6,9,12,15,18-24:5 (Figure 1D) was incubated with peroxisomes, microsomes and 1-acyl-GPC, there was a marked decrease in the production of acid-soluble radioactivity. The first cycle of β-oxidation of 6,9,12,15,18-24:5 also uses only the enzymes of saturated fatty acid degradation but, as soon as 4,7,10,13,16-22:5 is produced, its continued degradation requires both NADPH-dependent 2,4-dienoyl-CoA reductase and Δ9,Δ12-dienoyl-CoA isomerase, which is a component of the peroxisomal trifunctional enzyme [31].
two cycles of labelled arachidonate was esterified. The data clearly show that, both 24:4, 6,9,12,15,18-24:5 were incubated together for 30 min with peroxisomes, microsomes and 1-acyl-GPC. Because of chromatographic overlap problems, these studies were carried out by incubating 100 nmol of a labelled fatty acid with an equal amount of the appropriate unlabelled analogue. In two separate experiments it was observed that, when 100 nmol of [3-14C]24:5,n–6 was incubated with an equal amount of unlabelled 24:6,n–3, 12.2 and 8.5 nmol respectively of 22:5,n–6 was esterified after a 30 min incubation. In the direct crossover study using [3-14C]24:6,n–3 and unlabelled 24:5,n–6, 10.2 and 12.0 nmol respectively of 22:6,n–3 was esterified in two separate experiments. Under these conditions there were no differences in the amounts of 22:5,n–6 and 22:6,n–3 esterified when they were generated by co-incubation of their respective immediate C34 precursors.

**DISCUSSION**

When hepatocytes were incubated with 3-13C-labelled C34, n–3 fatty acids, only small amounts of the substrates were esterified directly into phospholipids. In the present study, as well as in an analogous study with labelled n–3 fatty acids [9], it was observed that these compounds were poor substrates for esterification into 1-acyl-GPC by microsomal acyl-CoA:1-acyl-GPC acyltransferase. When C34 acids are produced in the endoplasmic reticulum, their primary metabolic fate must be to move to another intracellular site for further metabolism. It remains to be determined whether they move as acyl-CoAs or whether they are hydrolysed in the cytosol [26,27] and reactivated at their site of subsequent metabolism. It also remains to be determined whether mitochondria, which lack a very-long-chain acyl-CoA synthetase [30], have the capacity to activate unsaturated C34 acids, convert them into acylcarnitines and subsequently degrade them completely, which would appear to be an example of futile metabolic cycling.

The results obtained here, as well as with the corresponding n–3 fatty acids [9], generally show that an inverse relationship exists between rates of peroxisomal β-oxidation and rates of microsomal esterification into 1-acyl-GPC. When [3-14C]24:4,n–6 was incubated with peroxisomes, the addition of microsomes and 1-acyl-GPC did not markedly decrease the production of acid-soluble radioactivity. The β-oxidation of 24:4,n–6 to 20:4,n–6 requires only the enzymes of saturated fatty acid degradation. Neither 24:4,n–6 nor 22:4,n–6, the product formed after one cycle of β-oxidation, was readily esterified into 1-acyl-GPC. By using 3H-labelled 24:4,n–6 it was shown that the primary metabolic fate of 20:4,n–6, when it was produced, was esterification rather than continued peroxisomal degradation. The first cycle of [3-14C]24:5,n–6 β-oxidation also uses only the enzymes of saturated fatty acid degradation. When [3-14C]24:5,n–6 was incubated with peroxisomes, microsomes and 1-acyl-GPC, there was a marked decrease in the production of acid-soluble radioactivity, which would appear to be determined during the second cycle of fatty acid degradation. As soon as 22:5,n–6 is produced, it is preferentially transferred out of peroxisomes and used as a substrate for esterification in microsomes rather than undergoing continued peroxisomal β-oxidation. Since peroxisomes do not contain acyl-CoA:1-acyl-GPC acyltransferase [8], the chain-shortened product must then be transferred back to the endoplasmic reticulum for use in membrane lipid biosynthesis.

It appears that the low activity of peroxisomal NADPH-dependent 2,4-dienoyl-CoA reductase is a major control point in the regulation of peroxisomal β-oxidation. When [1-14C]22:5,n–6 was incubated with peroxisomes, or generated from its C34 precursor, 2-trans-4,7,10,13,16-22:6 accumulated. Similar results were obtained with the analogous n–3 fatty acids, i.e. [3-14C]24:6,n–3 and [1-14C]22:6,n–3 [9]. When 3-14C-labelled 24:4,n–6 and 24:5,n–6 were incubated together in the presence of microsomes and 1-acyl-GPC, there was preferential esterification of 22:5,n–6 compared with 22:4,n–6. Similar results were obtained when 3-14C-labelled 24:5,n–3 and 24:6,n–3 were incubated together. When [1-14C]16:3,n–6, the product formed after two cycles of arachidonate β-oxidation, was incubated with peroxisomes, 2-trans-4,7,10-16:4 accumulated [9]. It was possible to detect labelled 4,7,10-16:3 in the...
medium when control fibroblasts, but not those from patients with Zellweger’s syndrome, were incubated with \(^{3}H\)-labelled arachidonic acid [34]. Collectively, the above findings suggest that, when an acid is produced in peroxisomes with its first double bond at position 4, its further degradation is impaired, since the next cycle requires the action of NADPH-dependent 2,4-dienoyl-CoA reductase. As a result, that fatty acid is preferentially transported out of peroxisomes, via some unknown pathway, for use in another subcellular compartment, such as for rapid acylation, as was observed when 22:5n−6 was produced from 24:5n−6.

It has generally been recognized that desaturation of linoleate and linolenate at position 6 is the rate-limiting step in the biosynthesis of long-chain n−6 and n−3 fatty acids [35–37]. When rats are raised on a chow diet, their liver phospholipids contain large amounts of arachidonate, but small amounts of 22:5, n−3. Conversely, the same membrane lipids contain little if any 20:5, n−3 and small amounts of 22:5n−3, but 22:6n−3 is a major component. Although the pathways for 22:5n−6 and 22:6n−3 biosynthesis are identical, no single reaction rate for any reaction can explain these compositional differences. Reaction rates for the desaturation of 24:4n−6 and 24:5n−3 at position 6 were similar. Moreover these rates were similar to those for the desaturation of linoleate and linolenate at position 6 [38]. Reaction rates for chain elongation of 20:4n−6 and 20:5n−3 were similar to each other, but about 5-fold greater than those for chain elongation of 22:4n−6 and 22:5n−3 [2]. None of these reaction rates in themselves can be used as reliable indicators to predict why membrane lipids accumulate specific fatty acids. In a similar way, the reaction rates for the esterification of analogous n−3 and n−6 fatty acids into 1-acyl-GPC were similar, and could not be used as predictors as to why membrane lipids accumulate specific unsaturated fatty acids [21]. In the study reported here, it was found that there was no selectivity in the degradation of 3-\(^{14}C\)-labelled 24:5n−6 and 24:6n−3 to their respective 1-\(^{14}C\)-labelled fatty acids followed by their movement out of peroxisomes for esterification into 1-acyl-GPC. The fatty acids 22:4n−6 and 22:5n−3 may be viewed as central intermediates in determining how much 22:5n−6 and 22:6n−3 is produced. Both 22:4n−6 and 22:5n−3, when produced in micromoles, may be esterified directly, partially \(\beta\)-oxidized to yield 20:4n−6 and 20:5n−3, which can be esterified, or metabolized into 24:5n−6 and 24:6n−3, which are esterified following chain shortening. When \([3-^{14}C]22:4n−6\) was injected into the tail vein of rats fed a chow diet, 96 and 4\% \(^{14}C\)-labelled fatty acids were produced.

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