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Inhibitors of Retinyl Ester Formation Also Prevent the Biosynthesis of 11-*cis*-Retinol†

Achla Trehan, Francisco J. Cañada, and Robert R. Rando*
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115

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ABSTRACT: Lecithin retinol acyl transferase (LRAT) from the retinyl pigment epithelium is potently inhibited by *all-trans*-retinyl α-bromoacetate in the micromolar range. The inhibition is competitive and reversible. The retinyl pigment epithelium also contains an enzymatic activity capable of converting added *all-trans*-retinol into 11-*cis*-retinol. This isomerization is likely to require the intermediate formation of *all-trans*-retinyl esters, which are themselves produced by LRAT action. Here this possibility is directly tested by studying the effect of *all-trans*-retinyl α-bromoacetate on the isomerization reaction. When pigment epithelium membranes are preincubated with *all-trans*-retinyl α-bromoacetate, they form neither retinyl esters nor 11-*cis*-retinol from added *all-trans*-retinol. However, if the pigment epithelium membranes are first allowed to form *all-trans*-retinyl esters from *all-trans*-retinol before the addition of *all-trans*-retinyl α-bromoacetate, then 11-*cis*-retinol formation proceeds at close to the rate found in the absence of inhibitor. In addition, 11-*cis*-retinyl esters are not formed under these conditions, eliminating the possibility of a direct ester–ester isomerization route. Therefore, *all-trans*-retinyl esters are obligate intermediates in the biosynthesis of 11-*cis*-retinol.

Membrane phospholipids have been identified as the energy source that drives the thermodynamically unfavorable trans to cis isomerization required in the biosynthesis of the visual chromophore 11-*cis*-retinal (Deigner et al., 1989). Using the energy of the phospholipids, retinal pigment epithelium membranes can process added *all-trans*-retinol (vitamin A) into 11-*cis*-retinol in the absence of soluble energy sources (Bernstein et al., 1987a,b). This reaction, which completes the visual cycle, appears to require the intermediate formation of *all-trans*-retinyl esters, which are then directly isomerized, with hydrolysis, to form 11-*cis*-retinol by an "isoesterase" enzyme (Deigner et al., 1989). The proposed energy transduction mechanism couples the free energy of hydrolysis of the retinyl esters, estimated to be −5 kcal/mol, to the energy-requiring trans to cis isomerization, known to be +4.1 kcal/mol (Rando & Chang, 1983), resulting in an overall exothermic reaction. Membrane phospholipids are involved in the reaction, because they are the acyl group donors in the synthesis of retinyl esters from vitamin A (Barry et al., 1989; Saari & Bredberg, 1989). In the latter transesterification reaction, the acyl group from the 1-position of lecithin (phosphatidylcholine) is transferred to retinol yielding a 2-acyl lysophospholipid and a retinyl ester. Hence, the enzyme is a lecithin retinol acyl transferase (LRAT).[1] The isomerization process as a whole demonstrates a new role for membranes—that of providing energy to drive otherwise unfavorable biochemical reactions.

Substantial circumstantial evidence has been presented which is consistent with the hypothesis that the actual isomerase substrates are *all-trans*-retinyl esters and not vitamin A itself. Vitamin A is rapidly converted into retinyl esters by pigment epithelial membranes prior to isomerization (Bernstein et al., 1987; Fulton & Rando, 1987; Deigner et al., 1989). Retinyl esters themselves, under appropriate conditions, can be processed by the membranes into 11-*cis*-retinol (Deigner et al., 1989). Quantitative cleavage of the C–O bond (Deigner et al., 1989) and inversion of absolute configuration at C-15

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[1] Abbreviations: RBA, *all-trans*-retinyl α-bromoacetate; PE, retinal pigment epithelium; LRAT, lecithin retinol acyl transferase; BSA, bovine serum albumin; DTT, dithiothreitol.

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of retinol (Law & Rando, 1988) have been shown to occur concomitant with isomerization. Finally, the retinyl ester synthetase and isomerase activities appear to copurify (Barry et al., 1989). If retinyl ester formation is obligate in the formation of 11-*cis*-retinol, then specific inhibitors of retinyl ester formation from vitamin A should act pleiotropically on isomerization so that they inhibit isomerization too. On the other hand, if vitamin A is directly isomerized into 11-*cis*-retinol, then inhibitors of retinyl ester synthesis should actually enhance isomerization, as less substrate would be lost to ester formation. In this paper it is demonstrated that *all-trans*-retinyl α-bromoacetate (RBA), which is shown here to be a potent, reversible inhibitor of LRAT, also blocks isomerization from added vitamin A. It is concluded that retinyl ester formation from *all-trans*-retinol is obligate in the formation of 11-*cis*-retinol.

Materials and Methods

Materials

Frozen eye cups devoid of retinas were purchased from J. A. and W. L. Lawson Co., Lincoln, NE. Zwittergent 3-14 detergent was a product of Calbiochem. DTT and fatty acid free BSA were purchased from Sigma. Bio-Beads SM-2 (20–50 mesh) is a product of Bio-Rad. *all-trans*-Retinol, retinal, and retinyl palmitate were purchased from Fluka. NaB^3H_4 was a product of Amersham.

Methods

Preparation of Retinoids. Standard mixtures of isomeric retinols and retinyl esters were prepared as described elsewhere (Bernstein & Rando, 1986). [15-^{3}H]-*all-trans*-Retinol of specific activity = 6.5 Ci/mmol was prepared by the NaB^3H_4 reduction of retinal. The synthesis of retinyl bromoacetate (RBA) was accomplished by the reaction of retinol with bromoacetyl bromide in the presence of pyridine, as described in the literature (Gawinowicz & Goodman, 1982). [15-^{3}H]RBA was prepared along the same lines, starting from [15-^{3}H]-*all-trans*-retinol.

Preparation of Bovine Pigment Epithelium Membranes. The isolation of the pigment epithelium membranes from bovine eye cups has already been described (Fulton & Rando, 1987). To obtain the detergent-solubilized membranes, a protein solution in 50 mM sodium phosphate buffer (pH 7.2) at a final concentration of 6–8 mg/mL was used. To 5 mL of this solution, 450 μL of Zwittergent 3-14 solution 1.2%, w/v) and 150 μL of a solution of DTT (0.8%, w/v) were added. A 150000*g* supernatant of the resulting solution was used for the assay. The protein determinations were done with micro-Lowry assays (Peterson, 1977).

Assay Conditions. Unless otherwise indicated, all assays were performed in the absence of light. The procedure for the analysis of ^{3}H-labeled retinoids has been described elsewhere (Bernstein et al., 1987). In a typical assay 0.6 μCi of [15-^{3}H]-*all-trans*-retinol (solvent evaporated under N_2 stream) was incubated with 200 μL of the pigment epithelium homogenate (protein concentration = 0.5 mg/mL) with BSA (0.5%, w/v) as the retinol carrier. The incubations were carried out at 37 °C. The reactions were quenched with methanol, and 400 μL of hexane was used for the extraction of the retinoids. The isomeric retinols and retinyl esters were separated by HPLC as previously indicated (Bernstein et al., 1987). Radioactivity was counted with an on-line Berthold LB 506-C HPLC radioactive monitor interfaced with an IBM XT computer. In inhibition studies, the membrane preparation was added to a solution of RBA sonicated for 1 min, and incubated at 37 °C for 15 min.

Table I: Concentration Dependence of the RBA on the Inhibition of LRAT[a]

LRAT source	[RBA] (μM)	retinyl esters[b]	13-*cis*-retinol	*all-trans*-retinol[b]
PE membranes	0	58.2 ± 0.6	4.3 ± 0.3	28.5 ± 1.1
PE membranes	0.5	14.5 ± 0.5	4.3 ± 0.0	75.8 ± 1.0
PE membrane	1.0	4.4 ± 0.8	3.7 ± 0.15	88.0 ± 1.0
PE membranes	2.0	1.9 ± 1.1	3.9 ± 0.3	90.1 ± 2.1
PE membranes	5.0	1.0 ± 0.2	4.0 ± 0.5	94.2 ± 1.3
solubilized enzyme	0	72.4 ± 1.1	2.1 ± 0.1	24.2 ± 0.6
solubilized enzyme	1.0	13.1 ± 0.6	4.0 ± 0.3	86.8 ± 1.1
solubilized enzyme	10.0	4.0 ± 0.2	4.2 ± 1.3	91.7 ± 1.9
solubilized enzyme	100.0	0.9 ± 0.4	5.1 ± 0.4	93.5 ± 1.2

[a] The incubation mixture included [15-^{3}H]-*all-trans*-retinol (0.5 μM), BSA (0.5%, w/v), and PE membranes or detergent-solubilized material at a final protein concentration of 0.5 mg/mL. The PE membranes or the detergent-solubilized material were preincubated with RBA for 10 min, after which substrate was added and the reaction was continued for 15 min. The amounts of the various retinoids are expressed as percent radioactivity incorporated in each product and were determined as indicated under Methods. [b] All values are mean ± SD.

Results

Inhibition of LRAT by all-trans-Retinyl α-Bromoacetate. Initial experiments were performed to determine in sensitivity of LRAT to varying concentrations of RBA (Table I). As seen here, even 0.5 μM inhibitor substantially inhibits the formation of retinyl esters from added *all-trans*-retinol, both in PE membranes and in detergent-solubilized solutions. In order to determine whether the mode of inhibition is reversible or not, solubilized LRAT was incubated with 5 μM RBA for 15 min, at which point the LRAT activity was almost completely abolished (3.5 ± 0.7% retinyl ester formation vs 74.1 ± 2.9% in the uninhibited control). Since the inhibitor is hydrophobic, it could be removed by extraction with Bio-Beads. After two washings with Bio-Beads, the RBA-treated sample esterified 57.4 ± 3.2% of the added *all-trans*-retinol, whereas control enzyme washed with the Bio-Beads esterified 62.5 ± 2.5% of the added substrate. Therefore, RBA is a reversible inhibitor of LRAT. This conclusion is also consistent with the observed lack of a time course for inhibition (unpublished experiments).

Since it has been reported that PE membranes contain a retinyl ester hydrolase activity (Blaner et al., 1987), it was of interest to assess the stability of RBA toward hydrolysis under the conditions of the incubations. When RBA was incubated with PE membranes, the RBA was hydrolyzed with a $t_{1/2}$ of approximately 2 h. Control membranes heated at 100 °C for 5 min only hydrolyzed the RBA to an extent of 10% after 2 h. It was of some interest to determine if the hydrolysis products of RBA, vitamin A, α-bromoacetate, and glycolic acid, could cause inhibition of LRAT. It was found that neither α-bromoacetate at 100 μM nor its hydrolysis product glycolic acid at 100 μM had any effect on the LRAT activity. In addition, 0.5 μM cold vitamin A had little or no effect on LRAT (unpublished experiments).

In addition to determining the effects of RBA on retinyl ester synthesis, it was also of interest to determine the inhibitor's effect on the stability of the retinyl esters once they had formed. To this end, PE membranes were preincubated with [^{3}H]-*all-trans*-retinol to allow for the in situ biosynthesis of [^{3}H]-*all-trans*-retinyl esters. One membrane sample was then treated with RBA, and a second membrane sample served as a control. As can be seen in Figure 1, in the untreated control a small increase in ester levels was observed, followed by a small decrease concomitant with the formation of 11-*cis*-retinol. Behavior identical with this has been previously ob-

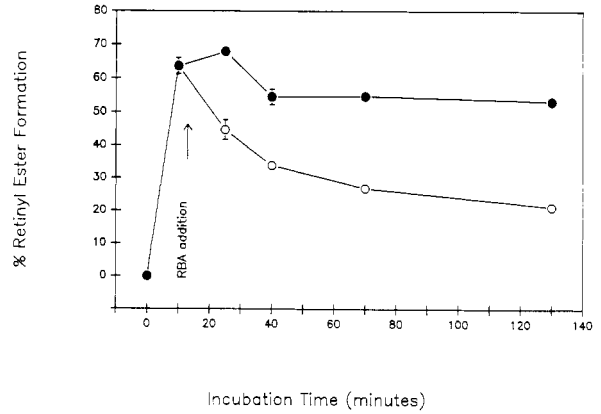

FIGURE 1: Effect of RBA on preformed retinyl ester levels. [15-^{3}H]-*all-trans*-Retinol (0.5 μM) was incubated with PE membranes (0.5 mg/mL) in the presence of BSA (0.5%) at 37 °C. After 10 min of incubation, an aliquot was removed for analysis, and the assay was divided into two portions. RBA (at a final concentration of 5.0 μM) was added to one portion of the assay. Both the mixtures were incubated again at 37 °C, and aliquots were removed after 15 min, 30 min, 60 min, and 1 h for the analysis of ^{3}H-labeled retinoids: (●) no RBA; (○) with RBA.

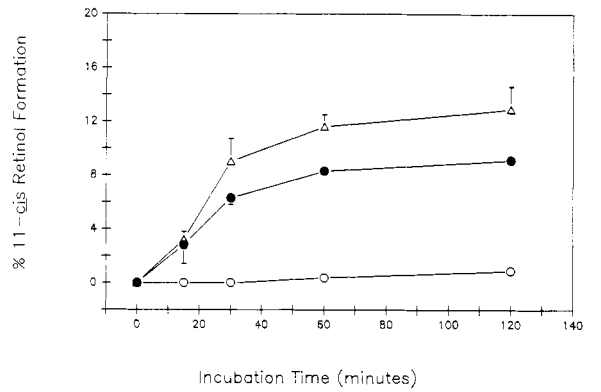

FIGURE 2: Inhibition of 11-*cis*-retinol formation by RBA. Time course of percent 11-*cis*-retinol formation (of the total pool): (○) when [^{3}H]-*all-trans*-retinol was incubated with membranes pretreated with RBA; (●) when RBA was added to the membranes after accumulation of antial amount of retinyl esters (10-min incubation); (△) a control (no RBA) of the experiment described with (●).

served (Bernstein et al., 1987b). On the other hand, in the presence of RBA the retinyl ester pool is substantially depleted—a result consistent with the presence of a retinyl ester hydrolase that is not inhibited by RBA.

Inhibition of 11-Cis Retinoid Formation by RBA. Since RBA is such a powerful inhibitor of retinyl ester formation, it was of some interest to determine the effect of the inhibitor on 11-cis retinoid formation from added *all-trans*-retinol. In the first set of experiments, PE membranes were preincubated with RBA for 15 min, followed by the addition of [^{3}H]-*all-trans*-retinol. In a second set of experiments, the order of addition was reversed, to allow for the formation of *all-trans*-retinyl esters. At this point in both sets of experiments, the formation of 11-*cis*-retinol was monitored (Figure 2). The results were quite clear-cut. When ester formation was allowed to precede the addition of RBA, substantial 11-*cis*-retinol formation occurred. When compared to control, in which no inhibitor was added, there was approximately 30% less 11-*cis*-retinol formed in the inhibited sample. It is noteworthy that 11-*cis*-retinyl esters were not formed under these conditions. Importantly, when RBA was added first followed by [^{3}H]-*all-trans*-retinol, no measurable biosynthesis of 11-*cis*-retinol occurred (Figure 3). Similar experiments were performed in which the formation of 11-*cis*-retinyl esters was

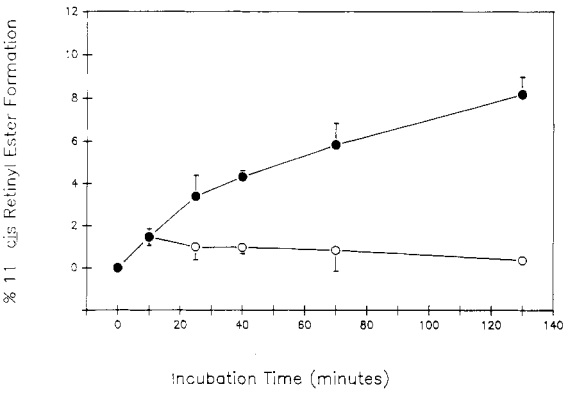

FIGURE 3: Effect of RBA on the formation of 11-*cis*-retinyl esters. The retinyl esters formed in an experiment performed as in Figure 1 were analyzed for the presence of different isomers. This figure shows the time couse of the 11-*cis*-retinyl ester formation (reported as percent radioactivity associated with 11-*cis*-retinyl ester, of the total pool): (●) no RBA; (○) with RBA.

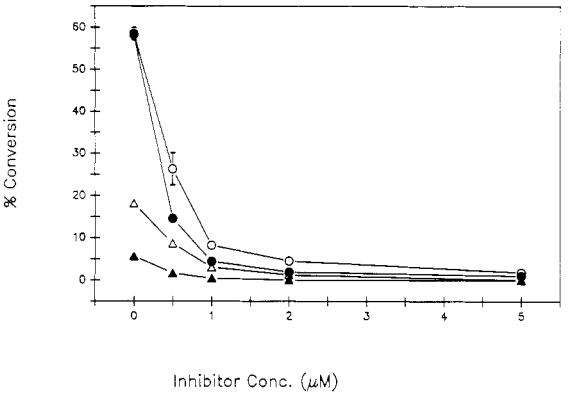

FIGURE 4: Concentration dependence of inhibition of RBA on retinyl ester formation and 11-*cis*-retinol formation. [15-^{3}H]-*all-trans*-Retinol (0.5 μM) was incubated with bovine PE membrane homogenate pretreated with RBA at different concentrations. The incubations were carried out at 37 °C for 1 h, and the products were analyzed after 15 min and at the end of the experiment (60 min) for the presence various ^{3}H-labeled retinoids. (●) Percent retinyl ester formation after 15 min; (○) percent retinyl ester formation after 60 min; (▲) 11-*cis*-retinol formation after 15 min; (△) 11-*cis*-retinol formation after 60 min.

followed rather than that of 11-*cis*-retinol (Figure 3). Pretreatment with inhibitor led to the almost complete abolition of 11-*cis*-retinyl ester formation as well.

Since RBA is slowly hydrolyzed during the incubations, it was of interest to determine if any of the possible hydrolysis products were themselves inhibitors of 11-cis retinoid formation. Neither bromoacetate nor its hydrolysis product, glycolic acid, had any effect at all on 11-cis retinoid formation when incubated at concentrations of 100 μM. The addition of cold *all-trans*-retinol at concentrations of 0.5 μM had no more than a 10% effect on 11-cis retinoid formation. Thus, the observed inhibition of 11-cis retinoid biosynthesis is due to a direct effect on the ester synthetase by RBA.

The experiments described above suggest a parallelism in the extent of LRAT inhibition and the extent of isomerization. This is shown graphically in Figure 4, wherethe percent LRAT inhibition is plotted against percent 11-*cis*-retinol formation at different concentrations of RBA. It can be clearly seen that the two processes are equally sensitive to inhibition by RBA.

Discussion

The experiments reported here were designed to determine whether retinyl ester formation is obligate in the biosynthesis

of 11-cis retinoids. Previous results from this laboratory have demonstrated that PE membranes contain the enzymatic capability for converting added *all-trans*-retinol into 11-*cis*-retinol(al) and 11-*cis*-retinyl esters (Bernstein et al., 1987a). However, prior to the formation of any 11-cis retinoid, the added *all-trans*-retinol is first processed (almost quantitatively) into *all-trans*-retinyl esters by LRAT (Bernstein et al., 1987b; Deigner et al., 1989). As already memtioned, accumulated circumstantial evidence points to a requirement for retinyl ester synthesis prior to isomerase action. In order to test the hypothesis that retinyl ester formation is obligate, a specific inhibitor of LRAT was required.

Previously, in a study of the effects of diverse relatively nonspecific agents including ethanol, *p*-(hydroxymercuri)-benzoate, and hydroxyl amine on the LRAT and isomerase activities it was found that the two enzymatic activities were affected in a roughly parallel manner by the reagents (Fulton & Rando, 1987). This suggested a functional linkage between the two enzymatic activities. However, because these reagents are relatively nonspecific in their actions, it was not fruitful to attempt to establish a more than phenomenological linkage between LRAT and the isomerase with them. A more specific inhibitor was desired. In fact, the work described here renders interpretable these earlier experiments with nonspecific reagents.

RBA has previously been shown to be a sluggish, apparently irreversible inhibitor of a retinol-binding protein from liver (Gawinowicz & Goodman, 1982). It is shown here that LRAT is powerfully and reversibly inhibited by RBA. Even at concentrations of RBA less than 1 μM, much of the LRAT activity was inhibited. That the observed inhibition is reversible can be shown by eliminating the inhibition after removal of the hydrophobic inhibitor by extraction with Bio-Beads. In addition, a time course for the inhibition of LRAT with RBA could not be established. LRAT inhibition occurs immediately after the addition of RBA. [It should be noted that simple aliphatic retinyl esters, such as the acetate and valerate esters, are not nearly as potent as RBA as inhibitors of LRAT, for reasons that are not clear (unpublished experiments).] RBA was slowly hydrolyzed by the PE membranes, producing *all-trans*-retinol and α-bromoacetate. These hydrolysis products, along with glycolic acid, had little or no effect on LRAT, showing that the observed inhibition was due to RBA itself. This is also consistent with the lack of a time course for inhibition. Attempts were made at synthesizing *all-trans*-retinylamine *N*-(α-bromoacetate) to overcome the susceptibility of RBA to hydrolysis. However, the amide proved to be extraordinarily unstable with respect to air oxidation, militating against its possible use here.

RBA proved to be exceedingly useful as an inhibitor of LRAT, as reported here. At micromolar concentrations, RBA basically abolished retinyl ester synthesis from exogeneous [^{3}H]-*all-trans*-retinol. Under these conditions, 11-cis retinoid biosynthesis did not occur. However, if *all-trans*-retinyl esters were allowed to form first, before addition of RBA, 11-*cis*-retinol synthesis occurred at close to the rate obtained in the complete absence of inhibitor. This showed that RBA has only a relatively minor direct effect on isomerase, as compared to its effect on LRAT. Indeed, the small diminution of the isomerization rate observed here could also be due to the decrease in retinyl ester levels caused by RBA (Figure 1). Thus, the inhibition of 11-cis retinoid synthesis observed when RBA was preincubated with PE membranes, before the addition of [^{3}H]-*all-trans*-retinol, was due to the inhibition of retinyl ester formation. If *all-trans*-retinol were the isomerase substrate, prevention of retinyl ester synthesis should have markedly enhanced the formation of 11-*cis*-retinol, rather than reduced it. This is because there would be much more *all-trans*-retinol available as a substrate for the putative retinol isomerase, as it would not be sequestered as ester. These results demonstrate both that *all-trans*-retinol cannot be the isomerase substrate and that *all-trans*-retinyl esters must be the isomerase substrate. In addition, 11-*cis*-retinyl esters were not formed from *all-trans*-retinyl esters in the presence of RBA, which eliminates the possibility that there is a direct ester–ester isomerization pathway. Therefore, 11-*cis*-retinol must be the direct product of isomerization.

In addition to the conclusion drawn above, the use of RBA revealed a hidden reaction in the PE membranes, namely, retinyl ester hydrolysis. In the presence of RBA, preformed retinyl esters are depleted to a significantly greater extent than can be accounted for by the formation of 11-*cis*-retinol (Figure 1). Since this depletion cannot be caused by LRAT running in reverse, this result almost certainly must mean that the retinyl esters are being hydrolyzed by a retinyl ester hydrolase whose activity is masked by the normally prodigious LRAT activity. Evidence for a PE-associated retinyl ester hydrolase activity has been described previously (Blaner et al., 1987).

The finding that retinyl ester formation is obligate in the formation 11-cis retinoids is completely consistent with all of the other evidence put forth to uncover the energy source capable of driving the thermodynamically unfavorable trans to cis isomerization crucial for rhodopsin regeneration (Deigner et al., 1989). This mechanism posits a fundamentally new role for membrane phospholipids; they can serve as an energy source that can be utilized in group-transfer reactions in much the same way that ATP can. It is unlikely that the discovery of this process in 11-*cis*-retinol biosynthesis is a singular occurrence. The use of lecithin acyl transferase enzymes of the LRAT type will doubtless be important in these reactions. Moreover, specific inhibitors of these enzymes will be of importance in establishing a physiological role for these enzymes. Hence, experiments involving RBA-type molecules may be of some general usefulness. Along these lines it will be of interest to determine whether the liver LRAT enzyme is inhibited by RBA and what the physiological consequences of this inhibition are.

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