Quantification, Characterization and Fatty Acid Composition of Lysophosphatidic Acid in Different Rat Tissues

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The amount and composition of lysophosphatidate present in different rat tissues have been estimated by an internal standard method in which a synthetic unnatural isomer (1-heptadecanoyl-*rac*-glycerol-3-phosphate) was added to the total lipid extracts, and the fatty acid composition of purified lysophosphatidate was determined. Lipids from tissues were extracted under acidic conditions, and the lysophosphatidate was purified by solvent partitions followed by thin-layer chromatography in multiple solvent systems. The purified lipid was shown to be 1-acyl-*sn*-glycerol-3-phosphate by chromatographic and chemical analysis, by its resistance to hydrolysis when treated with phospholipase A_2 and also by its complete conversion to 1-acyl-*sn*-glycerol when treated with alkaline phosphatase. The fatty acid consituents of this lipid were determined by gas-liquid chromatography of the derived methyl esters. The concentrations (nmol/g of tissue) of lysophosphatidate in various tissues were: 86.2 ± 4.2 in brain, 60.3 ± 6.3 in liver, 46.4 ± 6.5 in kidney, 30.6 ± 5.0 in testis, 22.3 in heart and 19.3 in lung. Mostly (80%) saturated fatty acids were found to be present in this lyso lipid. A significantly high level of stearic acid was present in this lipid from all the tissues (50–60% in liver, kidney, brain and testis, and about 40% in heart and lung) compared to palmitic acid (10–15% in liver, kidney and brain and 25–30% in testis, heart and lung). The fatty acid compositions of phosphatidic acid, the putative product of lysophosphatidate acylation, from different tissues were also determined and palmitate was found to be the major saturated fatty acid. These results suggest that tissue lysophosphatidic acid is not only formed by *de novo* biosynthesis but is also generated via the breakdown of phospholipids such as phosphoinositides.
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Lysophosphatidic acid (lysoPA) is a key intermediate for the biosynthesis of glycerolipids (1–3). This lipid is formed in three different subcellular compartments, either via direct acylation of glycerol-3-phosphate (G-3-P) in mitochondria and microsomes or via acylation of dihydroxyacetone phosphate (DHAP) in peroxisomes followed by reduction (2–4). This lyso lipid may also originate from the degradation of other lipids (5). The steady state concentrations of this important phospholipid in different animal tissues and its fatty acid compositions have not yet been reported in the literature. This is probably because during standard lipid extraction method (6), at neutral pH, this acidic lyso lipid is lost with the aqueous wash and therefore escapes detection (7). We recently developed a combined acid extraction and solvent partition method to isolate acyl DHAP from guinea pig liver and found that lysophosphatidate is extracted and partitioned in the same manner (8). After purification by TLC and analysis it was found that guinea pig liver contained fairly high level of lysoPA (140 nmol/g) and stearic acid was the major fatty acid component (66%) of this lipid. We also estimated lysoPA in three different rat tissues by enzymatically measuring the *sn*-glycerol-3-phosphate released after alkaline methanolysis of partially purified lipid extracts and found it to be relatively high (50–90 nmol/g) (8). However, in that work the loss incurred during purification of lysoPA was not considered and whether the lysoPA in rat tissues was also enriched in stearic acid had not been determined. Therefore, the present method of analysis of lysoPA in different tissues was developed where an internal standard was used to account for the loss of lysoPA during its extensive purification and for the quantitative determination and the fatty acid composition of this polar lipid. These results are reported here.

MATERIALS

Gas-liquid chromatographic (GLC) reference mixture of saturated fatty acid methyl esters of different carbon chains was purchased from Nu Chek Prep, Inc. (Elysian, Minnesota) and that of polyunsaturated fatty acids (PUFA, No. 2, animal source) was from Supelco, Inc. (Bellefonte, Pennsylvania) and used for the identification of unknown saturated and unsaturated fatty acids. n-Heptadecanoic acid was from Aldrich Chemical Co. (Milwaukee, Wisconsin); the methyl ester of this compound showed a single peak on GLC chromatogram. Authentic lipids including lysoPA, phosphatidic acid (PA), 1- and 2-palmitoyl-*sn*-glycerol, phospholipase A_2 from *Naja naja* venom and alkaline phosphatase from *E. coli* were obtained from Sigma Chemical Co. (St. Louis, Missouri). 1-Heptadecanoyl DHAP was prepared from n-heptadecanoic acid and glycolic acid as described before (9). 1-Heptadecanoyl-*rac*-G-3-P, which was used as an internal standard during the present studies for estimation of lysoPA, was made by reducing 1-heptadecanoyl DHAP by $NaBH_4$ in ethanol at pH 7.5 for 2 hr at 37°C as described before (8). This synthesized material gave one spot on TLC in different solvent systems and was found to contain only heptadecanoic acid as checked by GLC of the derived methyl ester. The fatty acid to phosphate ratio was 1:1. The phosphorus concentration of this material and other lipids was determined by the method of Ames and Dubin (10). Adult Sprague-Dawley male rats (200–250 g) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, Indiana) and fed standard diet (Purina Chow) and water ad libitum. Other materials were the same as described previously (8,9).

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METHODS

Extraction of lysoPA from tissues. Total lipids from liver, kidney, brain, testis, heart and lung of adult rats (150–200 g) were extracted by an acidic solvent extraction method (8). The rats were anesthetized with diethyl ether, the tissues were removed and immediately (within 15 sec) frozen in an isopentane/dry ice bath and, if necessary, stored at −70°C. The frozen tissues were weighed and quickly ground into small pieces with a pre-chilled (−20°C) mortar and pestle and immediately homogenized in chloroform/methanol/2M H_3PO_4 (1:1:0.05). During homogenization of the tissues, 25 nmol of synthetic 1-heptadecanoyl-*rac*-G-3-P per g of tissue was added to the homogenates. The lysoPA was separated from most other tissue lipids by partitioning the lipids between two phases, first at pH 4.4 where lysoPA was partitioned into the upper aqueous layer and then at pH 1.5 when lysoPA is extracted back into the lower chloroform layer by the methods previously described (8).

Purification of lysoPA. This partially purified lysoPA was further purified to homogeneity by using three consecutive thin layer chromatographic systems. The lipid extract was put as a band on a preparative TLC plate (E. Merck, Silica Gel-60, 0.5 mm thick) along with an authentic standard lysoPA spot side-by-side and the plate was developed in the first solvent system, chloroform/methanol/acetic acid/5% sodium bisulfite (100:40:12:4). The lipid bands were visualized under UV light after spraying the plate with Primuline (11). The lysoPA band was located by comparing its mobility to that of standard lysoPA (Rf = 0.33) and the content of this band was recovered by scraping it out and extracting the scrapings three times with chloroform/methanol (1:1) containing HCl (0.1M). The combined extract was washed with half volume of water to remove the acid. The lower layer containing the lysoPA was dried by blowing nitrogen, and the dried lipid was subjected to a second TLC using a basic solvent system i.e. chloroform/methanol/8 M NH_4OH (60:40:5). The lysoPA (Rf = 0.07) was recovered from the TLC plate as described above and then finally purified by TLC by using another solvent system of chloroform/methanol/acetic acid/acetone/water (100:30:20:40:10) (12). The lysoPA (Rf = 0.46) was extracted out from the TLC spot as described above and used for analysis.

Hydrolysis by alkaline phosphatase. About 50 μg of standard lysoPA or rat liver lysoPA (purified without the addition of the internal standard) dispersed in Tris buffer (0.15M, pH 8.5) by sonication was incubated with *E. coli* alkaline phosphatase (35 μg, 1.5 U) in a final volume of 0.5 ml. The mixture was incubated at 37°C for 2 hr. The product was extracted using an acidic Bligh and Dyer extraction method (13). When necessary, the progress of the reaction was studied by taking out an aliquot of the reaction mixture, extracting the lipids under acidic conditions (8) and separating them on TLC using chloroform/methanol/acetic acid/water (100:40:12:4). In this system the product monoacyl glycerol has an Rf of 0.88 and the Rf of lysoPA is 0.42.

Separation of monoacylglycerols. The positional isomers of monoacyl glycerol(1-*sn* and 2-*sn*) were separated from each other by TLC on silica gel containing boric acid (14). The boric acid impregnation was done by immersing the plates (5 × 20 cm Merck Silica gel-60) in methanolic boric acid (12% w/v) for 30 min. The plates were air dried for 15 min, activated at 110°C for 30 min and used immediately. By this procedure, the TLC plate was found to contain boric acid about 10% of the weight of the adsorbent. The monoacylglycerols were separated from each other by using a solvent system of chloroform/acetone (90:10). The respective Rf values for 1-palmitoyl and 2-palmitoyl-*sn*-glycerol were 0.12 and 0.20.

Extraction and purification of PA. The lower, washed chloroform layer which was saved during the lipid partitioning process (8) was concentrated under a stream of N_2. From this total lipid extract PA was isolated by two-dimensional TLC on a preparative plate (0.5 mm thick E. Merck Silica gel 60) using the solvent mixture of chloroform/methanol/8 M HN_4OH (17:7:1) in the first dimension followed by chloroform/methanol/acetic acid/water (80:40:7.4:1.2) in the second dimension essentially as described by Pumphrey (15). Purified PA migrated as a single spot having Rf of 0.04 in the first and 0.55 in the second solvent.

Treatment with phospholipase A_2. To 100 μg of PA or lysoPA dissolved in 0.5 ml of diethyl ether/methanol (98:2), 50 μl of a solution of lyophilized *Naja naja* venom (1 mg/0.5 ml of 0.3 M Tris-HCl, pH 7.5 containing 20 mM $CaCl_2$) was added. The mixture was vortexed vigorously for 30 sec and then incubated at room temperature for 3–4 hr with gentle magnetic stirring under N_2 essentially as described by Wells and Hanahan (16). After the reaction, most of the ether and methanol were evaporated off under N_2 and the product was dissolved in 2 ml of chloroform/methanol (1:1), followed by the addition of 0.8 ml of water containing 0.1M HCl. The lower chloroform layer was washed with chloroform/methanol/water (1:12:12). An aliquot of the lipid extract was put on TLC plate and developed with chloroform/methanol/acetic acid/water (100:40:12:4) for identification of the products by comparing the Rf values of standard compounds applied side by side. The Rf values of PA, lysoPA and free fatty acid in this solvent system were 0.77, 0.42 and 0.98, respectively.

Preparation and analysis of fatty acid methyl esters from lysoPA and PA. Fatty acid methyl esters were prepared from these lipids by alkaline methanolysis (17) with modification. In a screw-capped tube, a portion of lipid solution (~ 15 μg of lipid phosphorus) was dried down under N_2. One ml of 0.2 M NaOH in methanol and 2.0 ml of chloroform were added. The reaction mixture was mixed well and left at room temperature. After one hr of incubation, 0.75 ml of 0.35 N acetic acid was added, mixed well, and the phases were separated by centrifugation. Upper layer was removed and the lower layer was washed with 2 ml of 0.9% NaCl/methanol mixture (1:1). The methyl esters present in the final chloroform layer were purified by TLC using a solvent system of n-hexane/ether/acetic acid (50:50:2). The purified ester (Rf = 0.67) was extracted from TLC powder by three extractions with diethyl ether. After removing the ether by blowing N_2, the sample was dissolved in a small volume of n-hexane

and analyzed in a Hewlett-Packard gas chromatograph, Model No. 5710A, equipped with flame ionization detector and an electronic integrator (Spectra Physics). The stationary phase was 15% Silar 10C supported on Gas Chrom R in a glass-lined stainless steel analytical column (183 cm × 0.32 cm) and the temperature programming of 150-220°C was used for the GLC run. The components of methyl esters were identified by comparing the retention times with those of standards.

Estimation of lysoPA in tissues. The amount of lysoPA present in different tissues was evaluated from the relative amount of 17:0 in the fatty acid mixtures derived from the lipid. Because the endogenous 17:0 content of lysoPA isolated from different tissues was found to be very low (<1% of total fatty acids), it was assumed that all the 17:0 present in the final GLC analysis of the fatty acid composition of lysoPA was from the heptadecanoyl GP added (25 nmol/g tissue) during the lipid extraction process. This and the assumption that the proportional loss of this internal standard during purification is the same as the other molecular species of lysoPA were used to calculate the lysoPA content of different tissues. For example, nmol lysoPA/g tissue = [(100 − wt % 17:0)/wt % 17:0] × 25. The amount of each individual fatty acid present in lysoPA can also be calculated this way.

All other methods were the same as described previously (8,9). Lipids were stored, when necessary, at −70°C under N_2.

RESULTS

Isolation and characterization of lysoPA. The lysoPA purified by the above method migrated as a single spot with the mobility same as standard lysoPA in two other TLC systems: (a) chloroform/methanol/2-propanol/0.25% aqueous potassium chloride/ethyl acetate (30:9:25:6:18) (18) (Rf = 0.15) and (b) chloroform/methanol/acetic acid/water (100:40:12:4) (Rf = 0.42). When analyzed (8), the phosphorus/*sn*-glycerol-3-P/fatty acid molar ratio in different lysoPA tissue samples was found to be 1.0:1.0:1.0. From these criteria the isolated lysoPA was judged to be pure. The recovery of lysoPA from each TLC purification was 80-90% as measured by using radioactive lysoPA (8). The overall recovery of lysoPA from the crude lipid extract varies from 50-60% as indicated by the recovery of the internal standard (17:0) by GLC.

The lysoPA isolated from liver and kidney seemed to be 1-acyl isomer since it was not hydrolyzed by phospholipase A_2 as indicated by the absence of free fatty acid as a product when analyzed on the TLC plate and the total phosphorus content of lysoPA before and after the enzymatic treatment was the same. This is confirmed by hydrolyzing the lysoPA by alkaline phosphatase and analyzing the product. The lysoPA from liver, like the standard lysoPA, was completely hydrolyzed in 2 hr when treated with alkaline phosphatase as described in Methods. The resulting product, i.e., monoacyl glycerol, when chromatographed on 10% boric acid impregnated silica gel plate migrated as a single spot with authentic 1-palmitoyl-*sn*-glycerol. There was no spot corresponding to 2-palmitoyl-*sn*-glycerol.

TABLE 1

Lysophosphatidate Content of Different Rat Tissues[a]

Tissue	LPA (nmol/g of tissue) ± S.D.
Liver	60.3±6.3 (n=4)
Kidney	46.4±6.5 (n=4)
Brain	86.2±4.2 (n=3)
Testis	30.6±5.0 (n=3)
Heart	19.5, 25.1[a]
Lung	17.0, 21.6[a]

[a]The contents of LPA in different tissues of rat were estimated by GLC analysis of the fatty acid methyl esters including the internal standard methyl heptadecanoate (17:0) as described in the text.

n, Number of experiment; a, results of two experiments.

Tissue distribution of lysoPA. As shown in Table 1, the contents of lysoPA in different tissues of rat ranged from 20-90 nmol/g of tissue. Brain contained the highest level of this lipid (78-92 nmol/g of tissue) compared to other organs (20-60 nmol/g of tissue). Very little variation was observed in the lysoPA content of corresponding tissues from different animals. This is probably because of the use of adult animals of the same age group and using identical conditions to harvest the tissues. The amount of lysoPA present, however, is sufficient to isolate it from a small amount (0.5 g) of tissues and accurately analyze its fatty acid composition.

The presence of alkali stable lysoPA, i.e., lysoPA which may contain ether bond (instead of ester bond) was investigated by using rat kidney lysoPA. The phosphorus concentration before and after alkaline methanolysis of lysoPA was determined. The results indicated that about 10% of kidney lysoPA was alkali stable. The nature of this alkali stable lipid, however, was not investigated further. This is most probably alkyl G-3-P as any alkenyl G-3-P will be degraded by the repeated acidic extractions used to purify the lysoPA.

Fatty acid compositions of lysoPA from different tissues. Table 2 presents the fatty acid compositions of lysoPA from six different tissues of rat. Basically,

TABLE 2

Fatty Acid Compositions of LPA in Different Tissues of Rat[a]

Fatty acid	Composition in wt %					
	Liver	Kidney	Brain	Testis	Heart	Lung
14:0	9.5	5.2	0.5	tr	0.6	tr
16:0	16.0	13.6	9.9	31.5	26.1	28.4
18:0	50.6	59.9	51.3	56.6	35.6	42.6
18:1(n-9)	17.3	10.3	31.6	9.2	25.4	20.8
18:2(n-6)	5.2	6.9	tr	1.6	6.2	7.1
18:3(n-3)	—	—	5.0	—	1.5	1.0
20:4(n-6)	1.4	2.1	1.5	0.9	tr	tr

[a]The methyl esters were prepared from the purified LPA of different tissues containing 1-O-heptadecanoyl-rac-G-3-P as internal standard and analyzed by GLC as described in the text. The wt % of each fatty acid was calculated from the known amount of the internal standard (17:0). The data are the averages (ranges ± 3%) of three experiments.

tr, Trace, less than 0.1 %.

16:0, 18:0 and 18:1 were the three major components of lysoPA from all tissues. As can be seen (Table 2), 18:0 was the major fatty acid present in the lysoPA, e.g., more than half (50–60%) of total fatty acids were composed of 18:0 in the lysoPA isolated from liver, kidney, brain and testis. The ratio of 18:0/16:0 is also very high (3 to 5) in most of the tissues (Table 2). The fatty acid compositions of lysoPA from all these tissues were found to be very similar to guinea pig liver lysoPA which also contained very high amounts of stearic acid (65.9%) (8).

Purification and fatty acid composition of PA from different tissues. We also analyzed the fatty acid compositions of PA, the putative biosynthetic product from lysoPA, isolated from different tissues. Although the fatty acid compositions of rat liver and brain PA have been reported previously (19, 20), no information is available regarding the composition of PA of other organs. PA was isolated by two dimensional TLC using the same solvent systems as described by Pumphrey (15). An excellent and reproducible separation of this lipid from all other lipids was achieved; however, instead of the double spots for PA reported by Pumphrey (15), a single spot was found. This difference is probably because under the acidic lipid extraction used here, all the tissue phosphatidates were converted to the free acid form which migrated as a single spot on the TLC plate.

The amounts of PA present in various organs of rat, human and other animals have been reported by other workers (21—23). The fatty acid compositions of PA of different tissues of rat are presented in Table 3. It should be noted that, except in the case of brain and heart, the levels of 16:0 in the PA of different tissues were much higher than that of 18:0. For example, the ratio of 16:0/18:0 in brain was 0.53, which is lower than that in liver (1.48), kidney (1.25), testis (2.62) lung (1.31) and heart (0.91) (Table 3). The values of this ratio as calculated from literature reports are 2.2 (19) and 0.87 (20) for rat liver and brain, respectively. Among the mono- and polyunsaturated fatty acids, 18:1, 18:2 and 20:4 were the major components; however, in brain, a significant amount of 18:3 (6.0%) was also present.

Possmayer et al. have shown that rat liver PA contained 51% of 16:0 and 33% of 18:0 at the *sn*-1 position of glycerol (19). We determined the positional distribution of fatty acids in rat kidney PA by stereospecific hydrolysis with phospholipase A_2 and then analyzing the fatty acid composition of the resulting lysoPA. The results showed that 16:0 is present at a relatively higher amount (53%) than 18:0 (24%) at the *sn*-1 position of rat kidney PA.

TABLE 3

Fatty Acid Compositions of PA in Different Tissues of Rat[a]

Fatty acid	Composition in wt %					
	Liver	Kidney	Brain	Testis	Heart	Lung
14:0	1.8	3.8	tr	0.3	0.2	0.8
16:0	23.2	24.4	10.7	38.0	18.5	23.7
16:1(n-9)	4.0	3.7	0.8	2.2	2.1	3.5
18:iso	1.0	0.2	0.5	0.2	—	0.5
18:0	15.6	19.6	20.1	14.5	20.3	18.0
18:1(n-9)	20.1	12.2	33.0	17.5	14.6	15.7
18:2(n-6)	21.7	8.8	3.1	4.3	20.3	14.3
20:0	—	2.2	2.0	3.8	3.0	2.6
18:3(n-3)	1.5	0.2	6.0	—	—	1.6
18:4(n-3)	—	—	0.8	—	0.2	tr
20:2(n-6)	0.3	0.2	0.8	tr	0.4	0.5
20:3(n-6)	0.4	2.4	2.8	1.6	1.5	1.7
20:4(n-6)	6.3	10.0	9.7	9.5	6.6	6.6
20:5(n-3)	0.4	—	0.6	—	0.3	0.6
22:4(n-6)	—	0.9	2.8(?)	—	tr	0.9
22:5(n-3)	—	—	—	3.7(?)	—	0.7
22:6(n-3)	—	—	0.3	—	2.2	0.9

[a]PA from different tissues were isolated by two dimensional TLC and the total fatty acid contents were analyzed by GLC (see text). The data are the averages (ranges ± 5%) of two experiments.

tr, Trace, less than 0.1 %.

DISCUSSION

The use of the internal standard, 1-heptadecanoyl glycerol-3-P, was advantageous for the simultaneous quantification of the amount and fatty acid composition of the lipid. Since, like any other internal standard method a quantitative recovery of lysoPA was not necessary, the estimation was more easily done than any other method. This method should have a general applicability for the accurate analysis of any tissue lipid as the standards containing unnatural fatty acids can be easily synthesized by chemical or enzymatic methods.

As described above, lysoPA isolated from different tissues was found to be pure by chromatographic and chemical analysis. The lysoPA of rat liver was characterized as 1-acyl-*sn*-glycerol-3-P by the following criteria: (a) After alkaline methanolysis of the lipid, the products, i.e., fatty acid methyl esters and *sn*-glycerol-3-P formed were in 1:1 molar proportion. (b) Hydrolysis by alkaline phosphatase yielded 1-acyl-*sn*-glycerol as the sole lipid product. (c) The lipid is not hydrolyzed by phospholipase A_2. Van Deenen and de Haas (24) have shown that lysophosphatides (2-acyl) are hydrolyzed by snake venom phospholipase A_2.

Though the exact structure of lysoPA of organs other than liver was not determined, the similarity in the chromatographic migration rate with rat liver lysoPA, formation of 1:1 molar proportion of fatty acid and *sn*-glycerol-3-P after alkaline hydrolysis and the presence of mainly saturated fatty acids in the purified lysoPA (Table 2) indicate that this lipid in all tissues is predominantly 1-acyl-*sn*-glycerol-3-P.

The tissue concentrations of lysoPA, as reported here, are relatively high (20–90 nmol/g of tissue) compared to similar lipid biosynthetic intermediates such as acyl DHAP (2–10 nmol/g of tissue) (8) or CDP diacylglycerol (9–15 nmol/g of tissue) (25) but are lower than the corresponding amounts of phosphatidic acid present in these tissues (150–200 nmol/g of tissue) (21, 22). LysoPA is biosynthesized in three different subcellular compartments, i.e., mitochondria, peroxisomes and endoplasmic reticulum, but only in endoplasmic reticulum it is enzymatically acylated to PA (2, 3). This may account for the relatively high steady-state concentration of lysoPA in different tissues because the mitochondrial and peroxisomal-derived lysoPA must be transported to the endoplasmic reticulum for lipid biosynthesis. Also, from the high stearate content of

lysoPA (Table 2), it seems that a large fraction of tissue lysoPA originates from sources other than via the biosynthetic routes. This is because biosynthetically-derived lysoPA should have mostly 16:0, as both glycerophosphate acyltransferase and dihydroxyacetone phosphate acyltransferase have been shown to preferentially utilize 16:0 rather than 18:0 for the acylation of GP and DHAP, respectively (26-28). The predominance of 16:0 at the *sn*-1 position of PA as shown here and by other workers (19) also indicates that biosynthetic lysoPA should contain a relatively higher amount of 16:0 than 18:0. Therefore, our results suggest that a major fraction of lysoPA is probably formed via catabolic breakdown of lipids. One such possibility is the formation of this lipid from phosphoinositides which have mostly 18:0 at the *sn*-1 position (20, 29, 30). Lapetina and co-workers have shown that by the consecutive actions of phospholipase C, diacylglycerol kinase and PA-specific phospholipase A_2, the phosphoinositides are converted to lysoPA (5, 31). Therefore, it is possible that a portion of the tissue lysoPA originates from such receptor mediated breakdown of membrane lipids (32, 33). Further experiments such as the effects of chronic stimulation of phosphoinositide-linked receptor on the concentration and composition of lysoPA in cells would be useful to establish the source and function of this polar lipid in cellular metabolism.

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