Regulation of Membrane Lipid Synthesis in *Escherichia coli*

ACCUMULATION OF FREE FATTY ACIDS OF ABNORMAL LENGTH DURING INHIBITION OF PHOSPHOLIPID SYNTHESIS*

(Received for publication, January 17, 1975)

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SUMMARY

Glycerol starvation of an *Escherichia coli* glycerol auxotroph results in a specific inhibition of membrane phospholipid synthesis. Mindich ((1972) J. Bacteriol. 110, 96–102) observed only a trace accumulation of free fatty acid following glycerol deprivation. We have repeated these experiments using glycerol auxotrophs which also possess a lesion in \( \beta \) oxidation. This defect was introduced in order to control fatty acid degradation. In contrast to the previous results, we find free fatty acid does accumulate during glycerol starvation. Similar results were found using \( \beta \) oxidation-defective (\( \text{fad}^{-}\)) derivatives of both \( \text{gplA} \) and \( \text{plsB} \) glycerol auxotrophs. Upon glycerol starvation of a \( \text{plsB}^{-} \text{fad}^{-} \) strain, phospholipid synthesis is 90% inhibited. Following a lag of 20 to 40 min, free fatty acid synthesis begins and proceeds at a rate that steadily increases until the rate of fatty acid synthesis is equal to that found in glycerol-supplemented cultures. The accumulation of free fatty acid is the result of de novo synthesis. The average chain length of the fatty acid in the unesterified fraction is abnormally long. Two 20-carbon fatty acids, \( \text{cis-13-eicosenoic} \) and arachidonic acid, are found in this fraction. Furthermore, a greatly increased level of stearic acid and a small amount of a C-22 (behenic) acid are found in the free fatty acid fraction. These data indicate that acyl transfer into phospholipid is a major determinant of phospholipid acyl moiety chain length.

Other experiments have shown that the free fatty acid fraction in glycerol-starved cells is metabolically active. This fraction turns over despite the defective \( \beta \) oxidation system. Restoration of glycerol to starved cells allows the incorporation of the unesterified fatty acids into phospholipid.

The enzymology of membrane lipid synthesis has been studied most extensively in *Escherichia coli*. Although the enzyme chemistry of the synthetic pathways is well characterized (1), these studies have shed relatively little light on the regulation of these pathways (2). Very little free fatty acid is found during growth of a mutant deficient in fatty acid degradation (3). Fatty acid synthesis in *E. coli* therefore seems tightly coupled to phospholipid synthesis (3–5). The mechanism of this coupling is unknown.

Fatty acid synthesis is constitutive and is insensitive to repression (6, 7). Feedback inhibition therefore seems to be the most reasonable regulatory mechanism. An accumulated phospholipid precursor could inhibit an early step in fatty acid synthesis and thus coordinate the two pathways. In apparent agreement with this notion, Mindich (4) found that specific inhibition of phospholipid synthesis in *E. coli* (by starvation of a glycerol auxotroph for the required nutrient) caused a profound inhibition of fatty acid synthesis. Only trace amounts of free fatty acid were accumulated during the inhibition of phospholipid synthesis. However, similar experiments in two gram-positive bacteria gave completely different results. Glycerol deprivation of auxotrophs of *Bacillus subtilis* and of *Staphylococcus aureus* resulted in the accumulation of large amounts of free fatty acid of increased average chain length (4). Therefore, these gram-positive bacteria seemed to regulate fatty acid synthesis in a manner quite distinct from that of *E. coli*.

This conclusion was compromised by our finding that *B. subtilis* has much less ability to degrade fatty acids than does *E. coli*. Hence, the differing accumulation of free fatty acids observed between these two bacteria might be attributable to their degradation by *E. coli* and their lack of degradation by *B. subtilis*.

To test this hypothesis, we have constructed strains of *E. coli* that require glycerol (or \( \text{sn-} \)) for phospholipid synthesis and are also defective in fatty acid degradation. Upon glycerol starvation, these strains are able to synthesize fatty acid at the normal rate but accumulate these acids in a free (nonesterified) form rather than as phospholipid acyl moieties. In this paper we report several properties of this system.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—Strain LW1 is a \( \beta \) oxidation-negative (\( \text{fad}^{-}\)) derivative of strain BB20-36 of Bell (8). BB20-36 is a glycerol (or \( \text{sn-} \)glycerol 3-phosphate) auxotroph of *E. coli* K12 that owes its requirement to a lesion in the \( \text{plsB} \) locus. \( \text{plsB}^{-} \) mutants have a \( K_{m} \) defect in \( \text{sn-} \)glycerol 3-phosphate acyltransferase, the first

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* This investigation was supported by Grant AI-10186 from the United States Public Health Service and Grant GB-32063 from the National Science Foundation.
‡ United States Public Health Service Research Career Development Awardee I-K4-GM 70,411.

1 E. P. Gelmann, unpublished results.
enzyme of phosphatidic acid (hence phospholipid) biosynthesis. That is, for an sn-glycerol 3-phosphate is 10 fold higher for the mutant enzyme than for a normal enzyme (8). The &delta; oxidation (fadE), see Ref. 5) block was introduced by the mating technique of Cronan et al. (9), with the modification that the donor strain was counterselected by omission of methionine from defined media, and an improved phenocopy method was used (10). Induced cultures of LWI possess less than 1% of the &delta; oxidation activity observed in comparable cultures of BB26-36. The &delta; oxidation assay used was that of Klein et al. (5). Strain LW3 was constructed and tested exactly as described for LWI, except that the parent was Bell’s (8) strain BB20-14, which owes its glycerol (or sn-glycerol-3-phosphate) requirement to a lesion in the gpsA gene. The gpsA locus is the structural gene for the biosynthetic sn-glycerol-3-phosphate dehydrogenase of E. coli. All of the strains used are defective in glycerol catabolism (gpdD+). This lesion eliminates any carbon source “shift-down” effects that might be caused by glycerol deprivation.

Media and Growth Conditions— Cultures were grown at 37° in a standard medium consisting of Medium E (12) supplemented with 0.4% D-glucose, 0.1% casamino acids (vitamin free). When appropriate, glycerol was added to the standard medium to a final concentration of 0.02%. Glycerol deprivation was accomplished by filtration at room temperature on a membrane filter followed by 5 to 10 washes each with a few milliliters of the standard medium. The cells were resuspended by agitation in a volume proportional to the volume of the original culture medium. This cell suspension was then split into two flasks, one of which contained sufficient glycerol to give a final concentration of 0.02%. Growth was followed by turbidity measurement at 540 nm in a Coleman Jr. spectrophotometer or a Klett colorimeter.

Analysis of Radioactive Lipids—The incorporation of labeled acetate into lipid was assayed as previously described (13). All analyses were done by thin layer chromatography. Neutral lipids were separated from phospholipids either as previously described (13) or by development in petroleum ether/ether/acetic acid (70/30/2) for a distance of 18 cm from the origin, followed by development in CHCl3/CH3OH/acetic acid (95/25/8) for a 10-cm distance. The fractions were identified by co-chromatography of the appropriate standards.

Analysis of Neutral Lipid Fraction—The neutral lipid fraction was chromatographed in both the petroleum ether solvent system given above and the benzene/ethyl acetate/ether/acetic acid (80/10/10/02) solvent of Storrv and Tuckley (14). Standards of oleic acid, oleyl alcohol, monoglyceride, diglyceride, and &beta;-hydroxy fatty acids were co-chromatographed with the samples. Methylation Ester Formation—Either the dimethoxycarbonane method (9) or the diazomethane method described previously (9) was used. Fatty acids were released from phospholipids via saponification and extracted into ether following acidification of the hydrolysate (9).

Argentation Chromatography—Monoenoic esters were separated from one another (and from saturated esters) on thin layer plates impregnated with 50% AgNO3, as previously described (15). We found that &Delta;3 esters (of both the C-20 and the C-22 carbon acids) were well separated from (and ran ahead of) &Delta;2 esters such as cis-vaccenate. This result is in contrast to the behavior of &Delta;2 esters presented by Morris et al. (16) but consistent with the experimental results of Gunstone et al. (17). Generally, an internal standard of the methylest of arachidic, cis-15-eicosenoic, cis-vaccenic, and palmitoleic acids was added to each sample analyzed. The standards were visualized by spraying with dichorofluorescein (18) or anilinonaphtholsulfonic acid (19) and viewing under ultraviolet light. The radioactive compounds were located by autoradiography. In all cases the visible chromatogram and its autoradiogram could be superimposed.

Reversed Phase Chromatography—Plates impregnated with docosane were prepared, spotted with either saturated or unsaturated esters, and developed as previously described (18). These plates separate methyl esters according to chain length, the mobility being inversely proportional to the length of the acyl chain. Methyl esters of radioactive acids were used as standards and detected by autoradiography (chemical detection of compounds on such plates is difficult). The mobility of the various standards was found to agree very closely with our previous results (18) and with those of Bergelson et al. (20). Oxidation of Unsaturated Esters— Unsaturated esters were eluted to monoaarboxylic and dicarboxylic acids with periodate-permanganate and fractionated as described by Scheuerbrandt and Bloch (21). The chain length of the monoaarboxylic acid fractions was assayed by thin layer chromatography as described by Bergelson et al. (20).

Materials—Authentic fatty acid standards were from Nu-Chek Prep. Monoglyceride and diglyceride standards were from Serva. &beta;-Hydroxyacyl-CoA dehydrogenase was synthesized by hydridation (22) of trans-2-tetradecenoic acid (Research Organics). All radioactive isotopes were from New England Nuclear. The [14C]-methyl esters of cis-13-eicosenoic and arachidic acids were synthesized by overnight exposure to a mixture of 25 µl of [14C]-labeled methanol, 2 ml of hexane, 0.25 ml of 2-dimethoxypropane, and 1 µl of concentrated H2SO4.

RESULTS

Phospholipid synthesis is immediately inhibited by removal of glycerol from the medium of Escherichia coli glycerol auxotrophs (4, 8). Mindich (4) reported that only traces of free fatty acid accumulated under these conditions. As outlined above, however, these results could be explained by degradation of the free fatty acids as well as by an inhibition of fatty acid synthesis. To test this hypothesis, we examined glycerol auxotrophs which are also defective in the &delta; oxidation of fatty acids. Strain LWI is a glycerol auxotroph due to a defect in sn-glycerol-3-phosphate acyltransferase, the first enzyme of phospholipid biosynthesis. The defect in &beta; oxidation (fadE+) lowers the activity of this system to less than 1% of the normal activity.

In the first experiment, a log-phase culture of strain LWI was deprived of glycerol and briefly labeled with [14C]-acetate at various times after starvation (Fig. 1). The rate of total lipid synthesis was inhibited about 90% during the first 20 to 40 min after glycerol deprivation, but following this lag, the rate of total lipid synthesis increased exponentially until the normal (glycerol-supplemented) rate was reached (Fig. 1B). This result was in contrast to the experiments of Bell (8), in which a decreased rate of phospholipid synthesis was observed throughout the period of glycerol starvation. To reconcile our results with those of Bell (8) we supposed that most of the lipid accumulated during glycerol starvation was neutral lipid rather than phospholipid. Indeed, analysis of the labeled material showed that 70 to 90% of the label incorporated by glycerol-starved cultures was in free fatty acid (Fig. 1C). Free fatty acid was the only neutral lipid found and was identified by thin layer chromatography in two solvent systems (see “Experimental Procedures”), by formation of water-soluble potassium salts, and by conversion to methyl esters with diazomethane. No hydroxy fatty acids were found in this fraction. The accumulation of free fatty acid was much greater in LWI than in BB26-36, the fad+ parent of LWI (Fig. 2).

Our results differed markedly from those of Mindich (4). We attributed this difference to the block in &beta; oxidation present in our strain (Fig. 2). Another major difference between the two strains was the nature of the genetic lesion causing glycerol auxotrophy. Our strain was a glycerol auxotroph owing to a defective sn-glycerol-3-phosphate acyltransferase (the gpsA gene), whereas the auxotroph used by Mindich (4) was deficient in the biosynthetic sn-glycerol-3-phosphate dehydrogenase (the gpsA gene). To eliminate this disparity, strain LW3, a dehydrogenase-deficient (gpsA−), &beta; oxidation-deficient strain, was constructed and examined (Fig. 3). Strain LW3 synthesized free fatty acid during glycerol starvation in a manner similar to that observed for LWI, the fad− strain (Fig. 3). However, the lag before the beginning of free fatty acid synthesis appeared
FIG. 1. Growth, total lipid synthesis, and free fatty acid synthesis in strain LW1 in the presence and absence of glycerol. Strain LW1 growing exponentially in glycerol-supplemented standard medium was filtered, washed, and resuspended in the standard medium (see “Experimental Procedures” for details). Half of the culture was supplemented with glycerol; the other half remained unsupplemented. The cultures were shaken at 37°C and sampled at the indicated times. One-milliliter samples were removed from both cultures and added to a prewarmed tube containing 10 μCi of sodium 1-[14C]acetate (45 mCi/mmol). After 5 min of incubation, 6 ml of methanol/chloroform were added to quench the reaction. Lipid extraction was performed as previously described (13). The lipid fraction was separated into phospholipid and free fatty acid fractions as described under “Experimental Procedures.” A turbidity of 30 Klett Units equals a cell concentration of 1.5 × 10⁸/ml. A (growth curve): □, + glycerol; ○, − glycerol; B (synthesis of total lipids): □, + glycerol; ○ and ●, − glycerol (two different experiments); C (rate of free fatty acid synthesis): ○ and ●, − glycerol; □ and ■, + glycerol. The rates are expressed per 1.5 × 10⁸ cells. FFA, free fatty acid; gly, glycerol.

FIG. 2. Free fatty acid synthesis in glycerol-deprived culture of strains LW1 (fadE) and BB26-36 (fad+). The strains were grown in parallel deprived of glycerol, and labeled as described in Fig. 1. ●, LW1; ○, BB26-36.

FIG. 3. Growth, total lipid synthesis, and free fatty acid synthesis in strain LW3 in the presence and absence of glycerol. This experiment was performed as described in Fig. 1, except twice the amount of labeled acetate was used. Symbols, normalization, and abbreviations as in Fig. 1.

Analysis of Free Fatty Acid Fraction—The free fatty acid fraction and the fatty acid moieties of the phospholipid from both starved and unstarved cells were converted to methyl

esters and analyzed by argentation chromatography (Fig. 4). A compound running ahead of cis-vaccenate was found in the free fatty acid fraction but was not observed in either phospholipid fraction. The behavior of this compound in this chroma-
Fig. 4 (left). Analysis of the fatty acids from the free fatty acid and phospholipid fractions. A culture of strain LW1 was starved for glycerol and labeled with \[^{14}C\]acetate after 85 min of starvation exactly as described in Fig. 1 except that the amounts of culture and labeled acetate were increased 10-fold. The free fatty acid fraction and the phospholipid fatty acid moieties were converted to their methyl esters with diazomethane and chromatographed in the argentation system. The esters were identified as described under "Experimental Procedures." FFA, free fatty acid; PL, phospholipid; gly, glycerol.

Fig. 5 (right). Chain length determination of the saturated fatty acids from the free fatty acid and phospholipid fractions. The saturated fraction from the argentation chromatogram in Fig. 4 was eluted and chromatographed in a reversed phase system (see "Experimental Procedures"). The esters were identified by co-chromatography with authentic standards. Abbreviations as in Fig. 4.

Fatty acid ester

| Saturated | Fatty acid composition | \(R_p\) values (RPTLC) |
|-----------|-----------------------|------------------------|
| Palmitic   | 16.0                  | 18.2                   |
| Stearic    | 17.6                  | 18.0                   |
| Behenic    | 16.8                  | 18.2                   |
| Average chain lengths | | |
| SFA        | 16.0                  | 18.2                   |
| UFA        | 17.6                  | 18.0                   |
| SFA + UFA  | 16.8                  | 18.2                   |
| Ratio UFA/SFA | 1.18                  | 1.24                   |

* The abbreviations used are: gly, glycerol; PL, phospholipid; FFA, free fatty acid; UFA, unsaturated fatty acid; SFA, saturated fatty acid; RPTLC, reversed phase thin layer chromatography.

† The total counts chromatographed were 80,000, 70,000, and 145,000 cpm for the + gly PL, - gly PL, and - gly FFA samples, respectively.

The increased ratio of cis-vaccenic acid to palmitoleic acid in the phospholipid fraction of a glycerol-supplemented culture indicated a fatty acid composition similar to the phospholipid fraction from the same culture.

A much higher ratio of cis-vaccenic acid to palmitoleic acid than is usually observed in E. coli was found in the free fatty acid fraction and in both phospholipid fractions (Table I). The increased ratio in the phospholipids of a glycerol-supplemented culture is also observed in strain BB26-36, the plaB+ parent of LW1 but is not observed in strain B, the plaB- parent of BB26-36. The increased content of cis-vaccenate is therefore probably due to the plaB lesion in strains LW1 and BB26-36. Under the present conditions, cultures supplemented with glycerol may still be slightly starved for sn-glycerol 3-phosphate and thus accumulate longer chain lengths than normal (see above). The higher levels of free fatty acid observed in the glycerol-supplemented cultures of LW1 (Fig. 1) as compared to LW3 (Fig. 3) also suggest that glycerol-supplemented plaB+ strains are partially starved for glycerol. Further studies of this observation are in progress.

**Metabolic Activity of Free Fatty Acid Fraction**—Are the non-esterified acids which accumulate in glycerol-starved cells metabolically active? To answer this question we starved strain LW1 for glycerol in the presence of \[^{14}C\]acetate until labeled free fatty acid had accumulated. We then restored glycerol to the culture and simultaneously removed the \[^{14}C\]acetate. Following glycerol restoration, the label in the free fatty acid fraction decreased with an equivalent increase in the label found in phospholipid (Fig. 6). These acyl chains can therefore participate in phospholipid synthesis. It should be noted that more...
We have observed repeatedly that the amount of lipid synthesis in glycerol-starved cultures relative to the amount of synthesis in glycerol-supplemented cultures was dependent on the length of the labeling period. Short labeling periods (5 min or less) gave relatively more lipid synthesis than long labels (20 to 40 min). Since this effect was observed with tritiated water as well as with labeled acetate, a pool effect did not seem likely. The most reasonable interpretation of these data was that the free fatty acid accumulated during glycerol starvation turned over despite the $\beta$ oxidation block. To test this hypothesis, we did a "pulse chase" experiment on $^{14}\text{C}\text{acetate-labeled glycerol-starved cells. As shown in Fig. 7, addition of a large excess of nonradioactive acetate to such cells caused only a gradual cessation of $^{14}\text{C}\text{acetate incorporation. This result indicated that the pools of acetate-labeled fatty acid precursors in these nongrowing cells were quite large (growing cells chase quite efficiently (see Fig. 6)). After 10 min of chase, the amount of label in free fatty acid decreased with a half-life of about 30 min, indicating that the fatty acid was being degraded or altered. Our inability to chase the acetate pool efficiently implies that these data may be a considerable underestimation of the true rate of turnover. However, the finding that free fatty acid is lost in these cultures indicates that the dependency on labeling time can be attributed to turnover of this fatty acid.

Origin and Location of Free Fatty Acid Fraction-Several lines of evidence indicate that the free fatty acid fraction is the result of direct synthesis rather than of phospholipid synthesis followed by hydrolysis. First and most compelling, the chain length distribution and species of fatty acid found in the free fatty acid fraction differ greatly from those of the fatty acid found in the phospholipids of either glycerol-supplemented or nonsupplemented cultures of LW1. In fact, to our knowledge, fatty acids of 20 carbons have not been reported previously in E. coli. The accumulation of more than trace amounts of stearic acid is also unprecedented. Second, if a culture of LW1 is labeled before glycerol starvation (and acetate removal) no labeled free fatty acid is found upon starvation. Third, the relatively higher rates of acetate incorporation into free fatty acid seen as the labeling period is shortened (see discussion above) also suggest that the formation of these molecules is due to de novo synthesis.

The abnormally long fatty acids observed in the free fatty acid fraction (see below under "Discussion") are not the result of chain elongation of previously existing fatty acids. The 1-$^{14}\text{C}\text{-acetate-labeled monooxybolic acid derived from the methyl end of the 20 carbon unsaturated acid contains about 30% of the total radioactivity. This figure is the amount expected if the entire carbon chain is synthesized during the acetate labeling period.

The free fatty acid fraction is distributed between the cells and the medium. A portion (10 to 40%) of this fraction remains in the medium following cell removal by centrifugation or filtration.
DISCUSSION

The results reported in this paper lead to several conclusions concerning the regulation of lipid synthesis in E. coli. The accumulation of free fatty acid during glycerol starvation of either type of glycerol auxotroph strongly suggests that feedback inhibition of fatty acid synthesis (by an accumulated phospholipid precursor) is not responsible for the coupling between fatty acid and phospholipid synthesis seen in this organism. In this respect this gram-negative bacterium is similar to the gram-positive bacteria studied by Mindich (4).

The free fatty acid accumulated during glycerol starvation contains molecules of abnormally long chain lengths. An unsaturated fatty acid of 20 carbons in length and saturated fatty acids of 20 and 22 carbons are found. These fatty acids had not been reported previously in E. coli. The longer chain lengths normally found in this bacterium (stearic and cis-vaccenic acids) are also enriched in the free fatty acid fraction at the expense of palmitic and palmitoleic acids.

The in vivo fatty acid synthetase system of E. coli also tends to accumulate the longer of the normal fatty acid chains (for a review see Ref. 15). However, the synthetase system functions at only a small fraction of the whole cell rate and overproduces unsaturated fatty acids. The glycerol-starved cells synthesize fatty acid at the normal rate and produce a normal ratio of unsaturated to saturated species as free fatty acid (Figs. 1 and 2; Table I). The fidelity of our system, therefore, seems much superior to that of the fatty acid synthetase and thus allows more definitive studies of the regulation of phospholipid acyl group synthesis.

The predominance of abnormally long chain lengths in glycerol-starved cells indicates that a competition between fatty acid elongation and transfer of fatty acyl groups in sn-glycerol 3-phosphate determines the chain length of the acyl groups found in the E. coli phospholipid fraction. If acyl transfer is blocked by a lack of sn-glycerol 3-phosphate, then fatty acyl thioesters continue to be elongated and appear as free fatty acids of abnormal length. It was formerly believed that the determination of chain length in E. coli was solely a property of the fatty acid synthetase system. The belief was based on the work of Groenspan et al. (23) who showed that β-ketoacyl-acyl carrier protein synthetase was unable to elongate palmitoyl and cis-vaccenyl acyl carrier protein substrates although shorter chain lengths, both saturated and unsaturated, were readily elongated. The data presented in this paper indicate that acyltransfer is an important factor determining the exact chain length of the phospholipid acyl moieties. However, some chain length specificity must also reside in fatty acid synthesis (perhaps in the β-ketoacyl-acyl carrier protein synthetase) since the chains that accumulate during glycerol starvation are (at most) only about 30% longer than normal.

Two aspects of this study were unexpected. First, a lag before free fatty acid production began was observed consistently (Figs. 1 and 2). Glycerol starvation halted phospholipid synthesis immediately but production of free fatty acid commenced only following at least 20 min of starvation. Preliminary experiments suggest that protein synthesis is required during this lag to allow free fatty acid synthesis to commence and that the synthesis of these acids may be under catabolite control. Other preliminary results suggest glycerol starvation does not cause any gross changes in the intracellular acyl thioester levels. A second surprising result was the finding that intracellular free fatty acid turned over in spite of the β oxidation lesion carried by the strains. This turnover could be attributed to incorporation of free fatty acids into a chloroform-insoluble compound, but is more likely due to traces of β oxidation activity not detectable by the usual assay or to a fatty acid catabolic system separate from β oxidation. The recent discovery of traces of fatty alcohols in E. coli (24, 25) suggests a possible route for a nonoxidative catabolic pathway. However, no increase in the levels of long chain fatty alcohols were observed following glycerol starvation.

In conclusion, our results show that fatty acid synthesis can proceed normally in the absence of phospholipid synthesis. We have reported previously that fatty acid and phospholipid synthesis seem to be jointly regulated in experiments involving strains diploid for an unsaturated fatty acid biosynthetic gene (3). Comparison of these results with our present data suggests that the mechanism that prevents the accumulation of free fatty acid in normal cells regulates both fatty acid and phospholipid synthesis but is probably not evoked by an accumulation of intermediates in either pathway.

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J. Biol. Chem. 1975, 250:5835-5840.

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