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Major Integral Membrane Protein Immunogens of *Treponema pallidum* Are Proteolipids

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A number of the major pathogen-specific immunogens of *Treponema pallidum* were characterized recently as amphiphilic, integral membrane proteins by phase partitioning with Triton X-114 (J. D. Radolf, N. R. Chamberlain, A. Clausell, and M. V. Norgard. Infect. Immun. 56:490–498, 1988). In the present study, we demonstrated that the same membrane immunogens (designated as detergent phase proteins [DPPs]) become radiolabeled upon in vitro incubation of *T. pallidum* with various ^{3}H-labeled fatty acids. Radioimmunoprecipitation with a monoclonal antibody confirmed that the ^{3}H-labeled 47-kilodalton protein corresponded to the well-characterized treponemal antigen with the identical apparent molecular mass. Failure to detect ^{3}H-labeled DPPs following incubation with erythromycin confirmed that protein acylation required de novo protein synthesis by the bacteria. When treponemes were incubated with [^{3}H]myristate, [^{3}H]palmitate, or [^{3}H]oleate, radiolabeled proteins corresponding to the DPPs were detected upon autoradiography. Demonstration that a number of the abundant membrane immunogens of *T. pallidum* are proteolipids provides information to help clarify their membrane association(s) and may serve to explain their extraordinary immunogenicity.

Little information exists on the macromolecular structure(s) of *Treponema pallidum* subsp. *pallidum* (*T. pallidum*), the etiologic agent of venereal syphilis. Identification and localization of the membrane proteins of this fastidious spirochete have become major foci of treponemal research, because these proteins are likely to represent potential vaccinogens, targets of immune responses, and/or virulence factors. Recently, we demonstrated by Triton X-114 phase partitioning that a number of the major protein immunogens of *T. pallidum* are amphiphilic and therefore are likely to be integral membrane proteins (27, 28). Included among these membrane antigens (designated as detergent phase proteins [DPPs]) were the well-characterized 47-kilodalton (kDa) (5, 15, 17, 24) and 34-kDa (36, 37) immunogens.

Molecular cloning and nucleotide sequence analysis of the genes that encode the 47- and the 34-kDa proteins have facilitated structure-function analysis of these antigens (15, 24, 36; M. A. Swancutt, J. D. Radolf, and M. V. Norgard, unpublished data). Hydropathy analyses (18) derived from their amino acid sequences failed to identify unequivocal membrane-spanning domains in either of these proteins, findings which suggest that modifications, such as covalent attachments of lipids, may help to anchor them within treponemal membranes. Recent demonstrations of proteolipids in other bacterial pathogens (6, 9, 13, 38–40) provided additional impetus for these investigations. In this report, evidence is presented that the major integral membrane antigens of *T. pallidum* (27) contain covalently linked fatty acids.

MATERIALS AND METHODS

Bacterial strains. *T. pallidum* (Nichols strain) was passaged continuously in New Zealand White rabbits and extracted from testicular tissue by differential centrifugation (36). For some experiments, treponemes were purified further by Percoll density gradient centrifugation (12).

Immunologic reagents. Murine monoclonal antibody 11E3, directed against the 47-kDa antigen of *T. pallidum*, has been described elsewhere (5, 17, 21, 22, 24). Monoclonal antibody 11E3 was used to immunoprecipitate (24) the 47-kDa antigen from detergent phase preparations of *T. pallidum* labeled with ^{3}H-labeled fatty acids.

Radiolabeling reagents. *T. pallidum* proteins were labeled with Trans [^{35}S]label (1,165 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.), [9,10(*n*)-^{3}H]palmitate (54 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), [9,10(*n*)-^{3}H]myristate (39.3 Ci/mmol; Amersham), or [9,10(*n*)-^{3}H]oleate (10 Ci/mmol; Amersham). Tritiated fatty acids used to label *T. pallidum* were dried under nitrogen gas and suspended in a small volume of 95% ethanol (final ethanol concentration, <2.0%).

Intrinsic radiolabeling of *T. pallidum* proteins. Treponemes (10^{10}) isolated from tissue by differential centrifugation were incubated at 34°C for 20 h in 10 ml of labeling medium containing 100 μg of cycloheximide per ml (33, 34). Each label was added at a final concentration of 0.25 mCi/ml. Methionine and cysteine were omitted from the labeling medium when Trans [^{35}S]label was used. In one experiment, treponemes were purified by Percoll density gradient centrifugation after incubation with ^{3}H-labeled fatty acids. In another experiment, treponemes were preincubated for 1 h with 50 μg of erythromycin per ml (26, 35) at 34°C before addition of [^{3}H]palmitate. Radiolabeled *T. pallidum* was then extracted multiple times with organic solvents (described below) (1) or solubilized with Triton X-114 for phase partitioning (27). Before sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (19), samples were precipitated overnight in 10 volumes of cold acetone, centrifuged at 13,000 × *g* for 10 min, dissolved in 100 μl of 1% SDS, and reprecipitated with cold acetone.

Preparation of DPPs from *T. pallidum*. Extraction and

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phase separation with Triton X-114 were performed essentially as described by Radolf et al. (27). *T. pallidum* (labeled with either ^{35}S-labeled amino acids or ^{3}H-labeled fatty acids) was suspended in ice-cold phosphate-buffered saline containing 2% (vol/vol) Triton X-114 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and rotated end over end at 4°C overnight. Insoluble material was removed by centrifugation at 12,000 × *g* for 15 min at 4°C. The detergent extract was incubated in a 37°C water bath to effect partitioning into aqueous and detergent phases, which were then separated by centrifugation at 12,000 × *g* for 2 min at 4°C. The aqueous phase was removed, and the detergent phase was re-extracted five times with ice-cold phosphate-buffered saline.

SDS-PAGE. Samples to be analyzed on SDS-PAGE gels were boiled for 5 min in final sample buffer (62.5 mM Tris hydrochloride, 10% glycerol, 2% SDS) and electrophoresed through a 4% stacking gel and an either 10 or 12.5% resolving gel (19). The gels were fixed and enhanced for fluorography with 1 M sodium salicylate (3). Apparent molecular masses were determined by comparing the mobilities of polypeptides with those of molecular weight markers (Amersham).

Lipid analysis of ^{3}H-labeled fatty acids of whole *T. pallidum* and DPPs. To remove noncovalently bound lipids, either whole *T. pallidum* cells or DPPs (both labeled with ^{3}H-labeled fatty acids) were extensively extracted with organic solvents by the method of Bligh and Dyer (1) until radioactivity in the $CHCl_3$ phase was reduced to background levels. The final aqueous phases were dried under nitrogen, suspended in 4 N HCl, flushed with nitrogen, and heated at 100°C for 90 min. Samples were then adjusted to pH 12 with 4 N NaOH, flushed again with nitrogen, and heated at 100°C for 30 min. Each sample was acidified to pH 5 with 4 N HCl and then extracted twice by using organic solvents (1). The pooled chloroform phases were dried under nitrogen, derivatized (see below), and analyzed for fatty acids by high-performance liquid chromatography (HPLC).

HPLC analysis of ^{3}H-labeled fatty acids. ^{3}H-labeled fatty acids released from *T. pallidum* were derivatized to phenacyl esters by using dibromoacetophenone in the presence of 18-crown 6 ether catalyst (Alltech Associates, Inc., Applied Science Div., State College, Pa.) (10, 11). As an internal standard, 4 nmol of heptadecanoic acid (17:0), a fatty acid not found in nature, was added to each lipid extract before the sample was dried under nitrogen. Each derivatized sample was chromatographed isocratically on a C8 reverse-phase HPLC column (Beckman Instruments, Inc., Fullerton, Calif.) by using methanol-water (89:11 or 91:9 [vol/vol]) at a flow rate of 1 ml/min; the A_{254} was monitored. To measure radioactivity in disintegrations per minute, fractions were collected, dried, treated with fluor, and counted by liquid scintillation. Each sample was counted until a sufficient number of counts was recorded to ensure a counting error of less than 2%. The identity of each fatty acid was based on the mobility of its phenacyl derivative relative to that of fatty acid standards derivatized and chromatographed concurrently. Radioactivity was correlated with chromatographic A_{254} peaks.

RESULTS

Identification of proteolipids in *T. pallidum*. *T. pallidum* incorporated [^{3}H]palmitate into a limited number of proteins which were visualized by SDS-PAGE and fluorography (molecular masses, 65, 50, 47, 38, 36, 17, and 15 kDa; Fig. 1A, lane 3). With the exception of the 65- and 50-kDa proteins, all of these ^{3}H-labeled proteins partitioned into the detergent phase following extraction with Triton X-114 (Fig. 1A, lane 4). In whole treponemes (Fig. 1A, lane 3, and B, lane 2) and in the DPPs (Fig. 1A, lane 4), [^{3}H]palmitate radiolabeling of the 17-kDa protein was particularly intense. In contrast to the limited set of polypeptides labeled with ^{3}H-labeled fatty acids, a large number of treponemal proteins were labeled with ^{35}S-labeled amino acids (Fig. 1A, lane 1); as previously reported (27), only a small number of these proteins partitioned into the Triton X-114 detergent phase (Fig. 1A, lane 2). The relatively small number of proteins labeled with ^{3}H-labeled fatty acids, compared with labeling by ^{35}S-labeled amino acids, suggests that the fatty acids were not degraded before incorporation into the proteins.

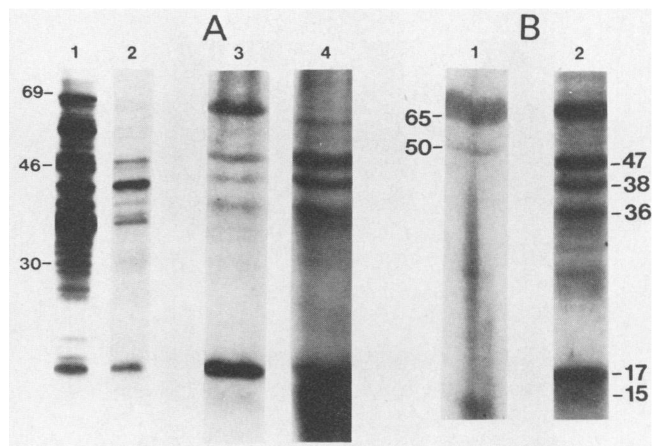

FIG. 1. SDS-PAGE and fluorography of [^{3}H]methionine- and [^{3}H]palmitate-labeled *T. pallidum* polypeptides before and after Triton X-114 phase partitioning. (A) *T. pallidum* (10^{10}) was intrinsically labeled with either [^{35}S]methionine (lane 1) or [^{3}H]palmitate (lane 3). A duplicate sample was extracted with Triton X-114 for analysis of [^{35}S]methionine- or [^{3}H]palmitate-labeled proteins (lanes 2 and 4, respectively). (B) Treponemes (3×10^9) were incubated with (lane 1) or without (1.5×10^9 treponemes; lane 2) erythromycin before addition of [^{3}H]palmitate. Molecular masses of proteins are expressed in kilodaltons.

It has been demonstrated that erythromycin is an effective inhibitor of protein synthesis in the Nichols strain of *T. pallidum* (26, 35). To support the notion that ^{3}H-labeled proteins were of treponemal origin, organisms were incubated with [^{3}H]palmitate with or without this antibiotic (Fig. 1B, lanes 1 and 2, respectively) (33–35). With the exception of the 65- and the 50-kDa proteins, the polypeptides previously labeled with [^{3}H]palmitate were no longer detectable. As an additional confirmation of the treponemal origin of the ^{3}H-labeled proteins, radiolabeled treponemes were purified further by Percoll density gradient centrifugation (12) before extraction with Triton X-114. Fluorography revealed the same ^{3}H- and ^{35}S-labeled polypeptides corresponding to the DPPs shown in Fig. 1A, lane 4 (data not shown). Neither whole Percoll-purified treponemes nor the DPPs contained the 65- or 50-kDa protein.

Radioimmunoprecipitation was used to confirm that the ^{3}H-labeled protein with an apparent molecular mass of 47 kDa was identical to the well-characterized 47-kDa *T. pallidum* membrane immunogen (5, 15, 17, 24). With monoclonal antibody 11E3 directed against the 47-kDa antigen, the ^{3}H-labeled 47-kDa protein was immunoprecipitated selectively from a Triton X-114 detergent phase extract (data not shown).

Fatty acid specificities of *T. pallidum* proteolipids. Fatty acids other than [^{3}H]palmitate were used in radiolabeling

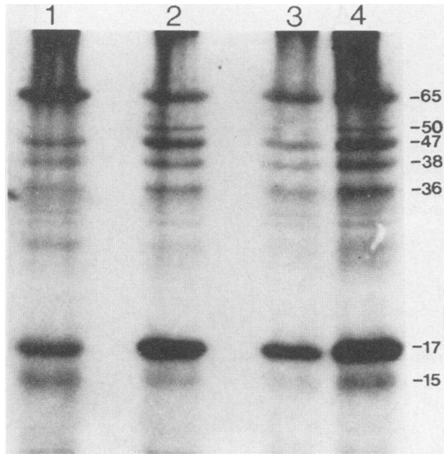

FIG. 2. SDS-PAGE analysis of *T. pallidum* proteins labeled with different long-chain fatty acids. *T. pallidum* cells (10^{10}) were incubated with [^{3}H]myristate (lane 1), [^{3}H]oleate (lane 2), or a mixture of [^{3}H]myristate, [^{3}H]oleate, and [^{3}H]palmitate (lane 4). Fluorograph of treponemal proteins labeled with [^{3}H]palmitate, as in Fig. 1, are repeated here for comparison (lane 3). Apparent molecular masses of the proteins are expressed in kilodaltons.

experiments to determine whether acylation of the proteins required particular long-chain fatty acids. Fluorography of treponemes incubated separately with [^{3}H]myristate, [^{3}H] palmitate, [^{3}H]oleate, or all three in combination showed a similar set of radiolabeled proteins (Fig. 2). In all cases, the 17-kDa antigen was labeled most intensely.

Analysis of covalently bound ^{3}H-labeled fatty acids. To confirm that the ^{3}H-labeled fatty acids were covalently bound to treponemal proteins and to identify the attached lipid moieties, ^{3}H-labeled fatty acid-labeled whole organisms and DPPs were extensively delipidated by extraction with chloroform-methanol and then hydrolyzed in acid to remove both (thio)ester- and amide-linked fatty acids. This was followed by re-extraction with chloroform-methanol and analysis of the organic phase by HPLC (Fig. 3). Material extracted into the organic phase contained 96% of the total radioactivity found in the prehydrolysis sample, providing additional evidence that the labeled fatty acids had not been substantially degraded and reincorporated into amino acids. When hydrolysate from whole treponemes labeled with both [^{3}H]oleate and [^{3}H]myristate was chromatographed, approximately 50% of the posthydrolysis radioactivity was recovered after HPLC; approximately 48% of this comigrated with myristate (14:0), and 5% comigrated with oleate (18:1) (Fig. 3C). Another peak (16% of the eluted radioactivity) comigrated with palmitate (16:0) (Fig. 3C). Incorporation of oleate was approximately one-half that of myristate when the specific activities of the two fatty acids used for labeling were compared. Identical results were obtained following acid hydrolysis of total [^{3}H]DPPs (data not shown).

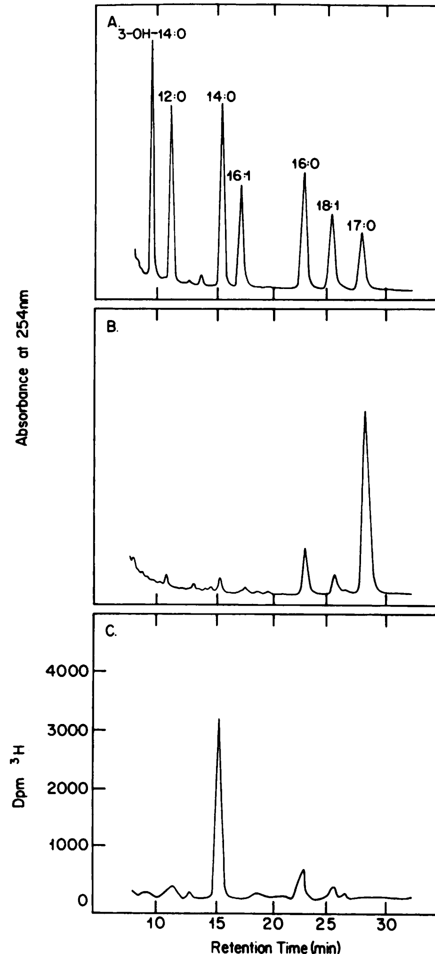

FIG. 3. HPLC of fatty acids from whole *T. pallidum* proteins labeled with a mixture of [^{3}H]oleate plus [^{3}H]myristate. (A) Fatty acid standards (120 pmol each): 3-OH-14:0, 3-OH-myristate; 12:0, laurate; 14:0, myristate; 16:1, palmitoleate; 16:0, palmitate, 18:1, oleate; 17:0, heptadecanoic acid (internal standard). (B) Fatty acids recovered after acid hydrolysis of *T. pallidum* (17:0 added as an internal standard); full-scale absorbance was 0.01. (C) Radioactivity recovered from the column during the separation shown in panel B.

DISCUSSION

In recent years, much emphasis has been placed on understanding the interaction of proteins with biomembranes. Attention has been given not only to polypeptide domains that anchor proteins within membrane bilayers but also to covalently attached lipids with analogous functions. In this regard, several proteins with covalently attached lipids (proteolipids) have been characterized in bacteria (6, 9, 13, 38–40). Braun lipoprotein, a 7.5-kDa peptidoglycan-associated outer membrane protein of *Escherichia coli*, represents the prototype for this class of bacterial membrane proteins (13). In this molecule, one fatty acid is amide linked to the amino-terminal cysteine, and a diacylglycerol is attached via a thioether bond to the same cysteine residue. The protein portion is initially synthesized as a higher-molecular-weight precursor (16) that is subsequently modified by signal peptidase II at a sequence consisting partly of Leu-Ala-Gly-Cys (39, 40). Other bacterial proteolipids undergo cleavage at an identical or closely related sequence (39, 40).

In the present study, we demonstrated that several of the major membrane immunogens of *T. pallidum* previously identified by phase partitioning with Triton X-114 (27) are proteolipids. Radioimmunoprecipitation analysis with a well-characterized monoclonal antibody directed against the 47-kDa membrane immunogen confirmed that this molecule was among the proteins containing radioactive, covalently

attached fatty acids. Although it was not readily discernible in our initial experiments (Fig. 1), we subsequently obtained evidence that the 34-kDa antigen of *T. pallidum* (36) is also a proteolipid. Experiments have shown that (i) *E. coli* or *E. coli* minicells readily incorporate [^{3}H]palmitate into a recombinant 34-kDa antigen expressed from the cloned gene; (ii) [^{3}H]palmitate remains associated with the recombinant DNA-derived molecule after extensive extraction with organic solvents; (iii) recombinant DNA-derived, [^{3}H]palmitate-labeled 34-kDa antigen can be selectively immunoprecipitated from *E. coli* by using relevant monoclonal antibodies; (iv) globomycin, a selective inhibitor of proteolipid processing in *E. coli*, inhibits processing of the molecule to the mature form in *E. coli*; and (v) globomycin also inhibits processing of the 34-kDa precursor molecule in *T. pallidum* (M. A. Swancutt, J. D. Radolf, and M. V. Norgard, manuscript in preparation). Furthermore, J. D. van Embden and co-workers have independently found and recently reported that the 34-kDa antigen (TpD) (37) is a proteolipid (L. M. Schouls, R. Mout, J. Dekker, and J. D. A. van Embden, Abstracts of the Biology and Pathogenicity of Treponemes, Birmingham, England, 11 to 13 April 1989). One of the less abundant DPPs (27, 28), the 17-kDa antigen, consistently demonstrated intenser ^{3}H-labeled fatty acid labeling, perhaps indicating a higher rate of metabolic turnover. This molecule and the 15-kDa protein most likely are identical to those previously reported by Lukehart et al. (20) and Hensel et al. (14) and further characterized by Radolf et al. (27, 28). The precise identities of the 36- and 38-kDa proteolipids remain uncertain, inasmuch as several possible candidates have been reported by Norris et al. (25). When treponemes were incubated with [^{3}H]palmitate with erythromycin (26, 35), only the 50- and 65-kDa proteins were detected. The 50- and 65-kDa proteins also were not detected in treponemes purified by Percoll density gradient centrifugation. Taken together, the findings indicate that these two proteins were most likely not of treponemal origin and that de novo protein synthesis by *T. pallidum* was required for acylation of the DPPs. Evidence for the covalent linkage of fatty acids to the proteins was found in that radioactive fatty acids remained bound to the proteins after multiple chloroform-methanol extractions, boiling in SDS, and subsequent analysis by SDS-PAGE. Hydrolysis in acid was required to cleave the fatty acids from the proteins. These results are consistent with conventional criteria for the identification of proteins with covalently attached lipids (31).

In other preliminary studies, three of the radiolabeled DPPs, the 47-, 38-, and 17-kDa proteins isolated within SDS-PAGE gels and treated by the method of Casey and Buss (2), were analyzed by sequential alkaline and acid hydrolyses to identify (thio)ester- and amide-linked fatty acids. For all three proteins radiolabeled with [^{3}H]palmitate, [^{3}H]myristate, or [^{3}H]oleate, all of the recovered radioactivity was obtained after alkaline hydrolysis and consisted almost entirely of the particular fatty acid used for labeling. Evidence for amide (alkali-stable) linkages was not found, suggesting that the fatty acids may be linked directly or indirectly to the proteins via ester bonds. These data cannot be considered definitive, however, because the treponemal proteins were labeled too inefficiently in these experiments. Conclusions regarding the precise structures of the proteolipids must await more rigorous biochemical analyses.

The finding that the 47-kDa antigen of *T. pallidum* is a proteolipid was somewhat surprising. Prior DNA and amino acid sequencing of the 47-kDa antigen (15) revealed no obvious consensus sequence for lipid attachment and processing by signal peptidase II, as described for other bacterial proteolipids (16, 39, 40). The 47-kDa antigen appears to lack a signal sequence of any known type (15), and in a companion report (4), it is shown that it does not undergo processing. One possible hypothesis derived from these data is that this molecule (and perhaps some of the other DPPs) may represent a previously undescribed type of procaryotic proteolipid. Bacterial proteolipids, therefore, may possess greater structural diversity than was formerly appreciated. Our findings may not be entirely unique, however, to *T. pallidum*; total membrane proteolipids of *Mycoplasma capricola* labeled with [^{3}H]oleate released 93% of the radioactivity upon exposure to mild alkaline treatment, suggesting the exclusive presence of ester linkages among these membrane proteins (8). Also, proteolipids in eucaryotes are known to be biochemically highly diverse with respect to their lipid moieties and the types of linkages utilized (32).

There is limited information regarding lipid metabolism in the virulent subspecies of *T. pallidum* (23, 30). *T. pallidum* apparently requires long-chain fatty acids for growth, as this organism appears not to synthesize, degrade, or desaturate fatty acids (23, 30). We found that fatty acids that varied in chain length from 14 to 18 carbons appeared to be incorporated, as were both saturated and unsaturated fatty acids. Although the minimal fatty acid chain length required for acylation was not determined, incorporation of [^{3}H]glycerol into proteolipids did not occur (unpublished data).

The findings of this study have important implications for efforts to determine the ultrastructure of this complex procaryote. Recently, cell fractionation studies have suggested that the *T. pallidum* outer membrane has a paucity of proteins (27). Freeze-fracture and freeze-etch electron microscopy studies have provided more direct support for this contention (29). However, the demonstration that a number of the abundant membrane immunogens are proteolipids complicates interpretation of these data, because proteins presumably anchored to membranes solely by lipid moieties would not be detected by freeze-fracture methods. The precise cellular locations for the protein components of these antigens, therefore, will need to be confirmed by other strategies. Our findings may also be relevant to the immunopathogenesis of treponemal infection. Is it coincidental that the major membrane immunogens of the organism possess a covalently bound lipid(s)? Studies have shown that lipid attachment may significantly enhance the immunogenicity of a protein (7). Investigations are under way to determine whether the extraordinary immunogenicity of the *T. pallidum* antigens described here is related to their proteolipid structure.

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