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Cell-Free Assembly of the Herpes Simplex Virus Capsid

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Herpes simplex virus type 1 (HSV-1) capsids were found to assemble spontaneously in a cell-free system consisting of extracts prepared from insect cells that had been infected with recombinant baculoviruses coding for HSV-1 capsid proteins. The capsids formed in this system resembled native HSV-1 capsids in morphology as judged by electron microscopy, in sedimentation rate on sucrose density gradients, in protein composition, and in their ability to react with antibodies specific for the HSV-1 major capsid protein, VP5. Optimal capsid assembly required the presence of extracts containing capsid proteins VP5, VP19, VP23, VP22a, and the maturational protease (product of the UL26 gene). Assembly was more efficient at 27°C than at 4°C. The availability of a cell-free assay for HSV-1 capsid formation will be of help in identifying the morphogenetic steps that occur during capsid assembly in vivo and in evaluating candidate antiherpes therapeutics directed at capsid assembly.

Infectious herpes simplex virus type 1 (HSV-1) consists of an icosahedral capsid containing the viral DNA and surrounded by a membrane envelope (5, 7, 18). The capsid is a protein shell approximately 15 nm thick and 125 nm in diameter whose major structural features are 162 capsomers (150 hexons and 12 pentons) that lie on a T = 16 icosahedral lattice. Hexons and pentons differ in their order of rotational symmetry but are otherwise morphologically similar. Each consists of a cylindrical protrusion (~10 nm in diameter) which extends radially outward ~11 nm from a floor layer (4 to 5 nm thick) where capsomers make contact with each other (3, 5, 7, 18, 19). The major capsid protein, VP5 (molecular weight [MW], 149,075), is the predominant structural subunit of both the hexons and the pentons; hexons contain six copies each, while pentons have five (12, 23). The capsid contains significant amounts of three other proteins, VP19 (MW, 50,260), VP23 (MW, 34,268), and VP26 (MW, 12,095). VP26 is found at the distal tips of the hexons (4), while VP19 and VP23 together form trigonal nodules called triplexes, which lie above the capsid floor and connect capsomers in groups of three (2, 12).

During infection by HSV-1, DNA-free progeny capsids, called B capsids, assemble in the infected cell nucleus and are later filled with DNA (1, 14–18). Formation of the B capsid involves the participation of two proteins, the scaffolding protein (VP22a) and the maturational protease (the product of the UL26 gene), in addition to the four structural polypeptides mentioned above. The scaffolding protein is found inside the cavity of the B capsid, but it is not present in the mature virion. It is thought to be lost at or near the time DNA enters the capsid shell. In an effort to clarify the pathway of HSV-1 capsid assembly, we have recently described a system in which the process can be studied with a limited number of HSV-1 proteins (22). Six recombinant baculoviruses were prepared, each coding for one of the proteins involved in HSV-1 capsid assembly. The proteins were the six mentioned above, namely, the four structural proteins plus the maturational protease and the scaffolding protein. When insect (Sf9) cells were infected simultaneously with all six baculoviruses, HSV-1 capsids were found to assemble in the cell nucleus. We have taken the view that this system could be most productively employed to analyze the process of capsid assembly if assembly were to take place in the absence of living cells—in a cell extract. To construct an active cell-free system, we infected Sf9 cells separately with each of the six recombinant baculoviruses described above and prepared cell extracts from each. The extracts were then mixed and examined for the presence of HSV-1 capsids.

The method of preparation for the six recombinant baculoviruses is described by Thomsen et al. (22). Each recombinant baculovirus contains the gene for one of the HSV-1 proteins required for capsid assembly cloned into the polyhedron site of the baculovirus (*Autographa californica* polyhedrosis virus) genome. The procedures described by Summers and Smith (20) were employed for preparation of baculovirus stocks and for infection of Sf9 cells. Separate cell cultures were each infected (multiplicity of infection of 5) with one of the six recombinant baculoviruses and incubated at 28°C for 64 h. Cells were then harvested by centrifugation, suspended in an equal volume of phosphate-buffered saline (PBS), and lysed by several cycles (6 to 8 times) of freezing and thawing. The total protein concentration of most extracts was in the range of 15 to 22 mg/ml. Aliquots (usually 50 μl) of each extract were mixed, centrifuged briefly (16,000 × *g* for 1 min), adjusted to a concentration of 3% polyethylene glycol 6000, and incubated for 12 h at 27°C to allow capsids to form. Assembly products were harvested by ultracentrifugation as described in the legend to Fig. 1.

Electron microscopic examination of the reaction products (Fig. 1a to c) showed that they included a uniform population of closed structures with an obvious resemblance to native HSV-1 capsids (Fig. 1d). Their measured diameter, for instance, was 112.9 ± 5.5 nm (n = 35) while that of native B capsids prepared for electron microscopy in the same manner (i.e., by negative staining) was 119.5 ± 5.5 nm (n = 45). In images where the thickness of the capsid wall could be determined, it was found to be 14.4 ± 1.3 nm (n = 12) and 14.1 ± 1.5 nm (n = 12), respectively, for cell-free and native B

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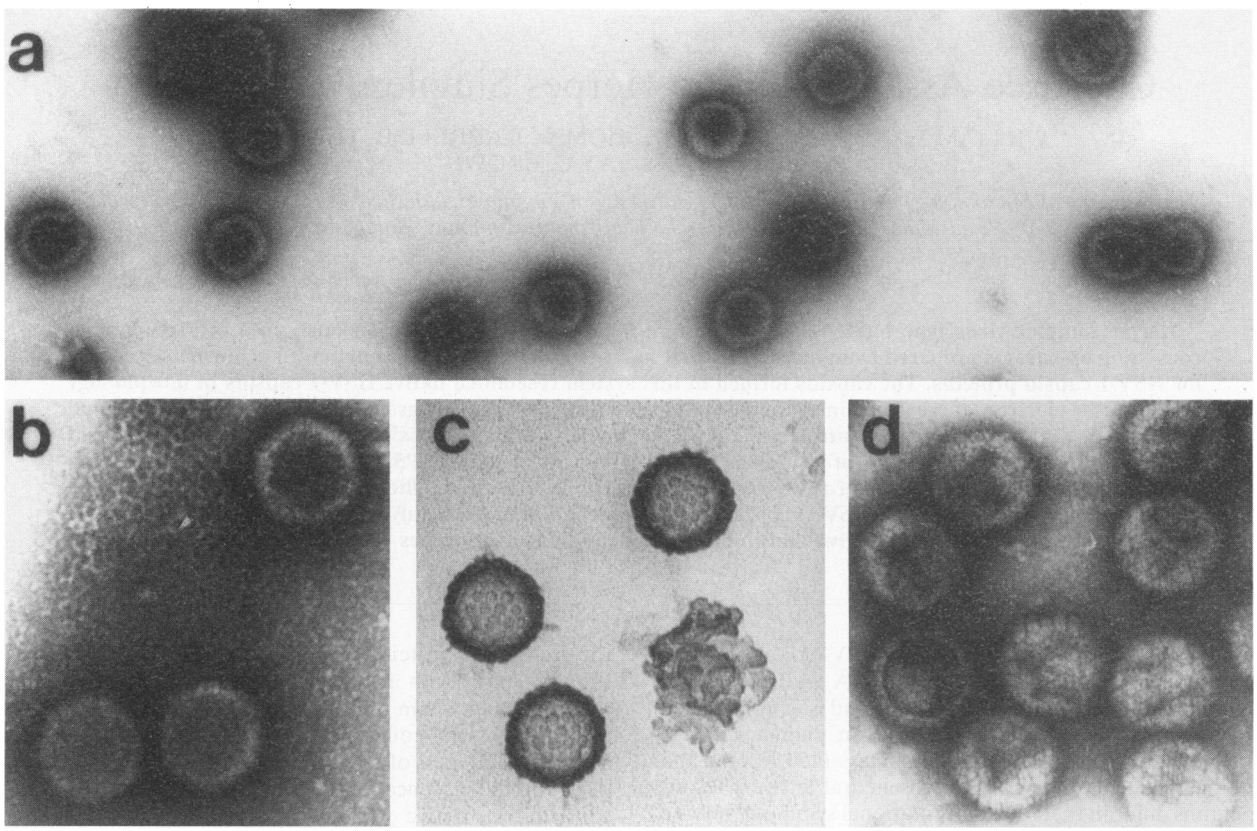

FIG. 1. Electron microscopic analysis of HSV-1 capsids formed in a cell-free system (panels a to c) and control HSV-1 capsids (panel d). Extracts of baculovirus-infected Sf9 cells were prepared, mixed, and incubated to permit capsid formation, as described in the text. To concentrate the products of cell-free assembly, the reaction mixtures were diluted to a volume of 500 μl with PBS, made 1% in Triton X-100, layered on top of a 100-μl cushion of 30% sucrose in TNE buffer in a 10- by 41-mm (0.8-ml capacity) tube, and centrifuged for 45 min at 22,000 rpm in a Beckman SW50.1 rotor operated at 4°C. The products of capsid assembly, which were found in a pellet at the bottom of the tube, were resuspended in 50 μl of TNE, layered on a linear 20 to 50% sucrose gradient (TNE buffer) prepared in a 5-ml SW50.1 ultracentrifuge tube, and centrifuged for 45 min at 22,000 rpm. Capsids (Fig. 2a, bottom band) were removed from the gradient, centrifuged into a pellet, resuspended in 50 μl of TNE, adsorbed to standard carbon-Formvar-coated electron microscope grids and prepared for electron microscopy by negative staining with 1% uranyl acetate (panels a, b, and d) or by critical-point drying and rotary shadowing (panel c) as previously described (10, 21). HSV-1 B capsids (shown in panel d) were prepared from HSV-1-infected BHK cells as described by Newcomb and Brown (9). Bar, 100 nm. Panels b, c, and d are shown at the same magnification.

capsids. Individual capsomers were resolved in both negatively stained and shadowed preparations, and they were seen to lie on a T = 16 icosahedral lattice (Fig. 1b and c). As in authentic HSV-1 capsids, capsomers in the cell-free products were found to extend radially outward from the capsid floor, to be cylindrical in shape, and to have an axial channel that extends (apparently) entirely through the capsid wall. The latter features of capsomer morphology can be seen in the capsomers at the edges of the capsids shown in Fig. 1b. In images where it could be assessed, the cavity of capsids assembled in the cell-free system appeared to be empty or nearly so. There was no evidence of the large mass of scaffolding protein (VP22a) found in the cavity of the B capsids isolated from HSV-1-infected cells (2, 6, 9).

Capsid formation was found to be dependent on the presence in reaction mixtures of extracts containing VP5, VP19, VP23, and the combination of VP22a and the maturational protease. As shown in Table 1, no significant assembly was observed unless all were present. Capsid yield was greatest when reaction mixtures contained the maturational protease, but some morphologically normal capsids appeared in its

TABLE 1. Capsid formation in the standard cell-free extract and in standard extracts lacking specific capsid proteins[a]

Protein(s) deleted	No. of capsids per electron microscope field
None (control)	168 ± 50 (n = 4)
VP5	0 (n = 12)
VP19	0 (n = 15)
VP23	0 (n = 12)
VP22a, protease	0 (n = 12)
Protease	26 ± 12 (n = 4)
VP26	157 ± 46 (n = 4)

[a] Standard reaction mixtures contained the following amounts of the six baculovirus-infected cell extracts: 50 μl each of VP5 (UL19), VP19 (UL38), VP23 (UL18), and VP26 (UL35), 20 μl of protease (UL26), and 50 μl of VP22a (UL26.5). These were mixed and incubated as described in the text, except that no polyethylene glycol was added. After incubation, capsids were harvested by centrifugation into a pellet as described for Fig. 1. Pelleted capsids were resuspended in 500 μl of TNE, applied to electron microscope grids, and stained with 1% uranyl acetate (21). The results reported are the numbers of complete capsids (± standard deviations) observed in representative electron microscope fields at a magnification of ×10,000.

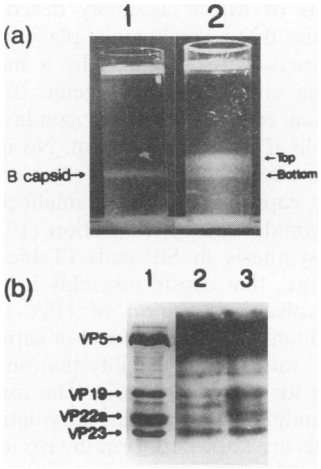

FIG. 2. Sucrose density gradient (a) and Western blot (b) analysis of capsids formed in the cell-free system. (a) Lane 1, HSV-1 B capsids; lane 2, products of cell-free assembly. (b) Lane 1, control HSV-1 B capsids (Coomassie blue stain); lane 2, top band from sucrose gradient; lane 3, bottom band from sucrose gradient. Capsid assembly was carried out as described in the text, except that the reaction mixture contained 50 mM EDTA and polyethylene glycol was omitted. The six extracts of baculovirus-infected cells were present in the same proportions shown in Table 2, but the total reaction volume was 800 μl. After incubation to allow capsids to form, reaction products were harvested by centrifugation through a layer of 30% sucrose and purified by sucrose density gradient ultracentrifugation as described for Fig. 1. A companion gradient contained HSV-1 B capsids. Top and bottom bands of reaction products were removed from the gradient with a Pasteur pipette, concentrated by centrifugation into a pellet (22,000 rpm for 1 h at 4°C in an SW50.1 rotor; 0.8-ml tube), resuspended in 10 μl of TNE buffer and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting as described by Ye et al. (26). Blots were probed with a mixture of polyclonal (rabbit) antisera specific for VP5, VP19, VP22a, VP23, and VP26. All sera but the VP26 were a gift from G. Cohen and R. Eisenberg (24); VP26 antisera were the gift of D. McNabb and R. Courtney (8). VP23 and VP26 antisera were employed at a dilution of 1:500. All others were diluted 1:1,000. Protein bands were visualized by staining with ^{125}I-labeled staphylococcal protein A followed by autoradiography (panel b, lanes 2 and 3).

absence (see Table 1). In contrast, capsid formation did not require the presence of VP26.

As a measure of the overall efficiency of capsid formation in the cell-free system, we determined the fraction of the starting VP5 recovered in the capsid fraction after cell-free assembly took place. Capsids were allowed to form in standard reaction mixtures as described in Table 1. Capsids and capsid-related structures were then sedimented into a pellet as described in the Fig. 1 legend, and the protein composition of the pelleted material was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by staining with Coomassie blue. Quantitative measurements of the amount of VP5 present were made by laser densitometry of the stained bands (12). In two similar experiments, the amount of VP5 recovered in the pellet amounted to an average of 62% of the VP5 present in the original reaction mixture. As shown in Fig. 2a and the accompanying discussion below, this material is expected to be approximately an equal mixture of complete, closed capsids and incomplete, capsid-like structures. Thus, approximately 30% of the VP5 present in the original reaction mixture was recovered as completed capsid shells.

Assembly required that the reaction mixtures be warmed.

TABLE 2. Capsid formation in standard and modified cell extracts[a]

Modification	Capsid yield
None (standard reaction)	++
Incubate at 4°C	−
Omit PEG[b]	++
Omit PEG, add 50 mM EDTA	++
Add 10 mM cycloheximide[c]	++
Add DNase and RNase[d]	++
Add 100 mM dithiothreitol	−
Add 0.5% Triton X-100	+[e]

[a] Standard, unmodified reaction mixtures contained the same amounts of the six baculovirus-infected cell extracts as described in Table 1, except that the amount of the VP5 extract was 75 μl. Extracts were mixed in the presence of 3% polyethylene glycol 6000 plus antibiotics (100 U of penicillin and 100 μg of streptomycin per ml) and incubated at 27°C for 12 h as described in the text. Capsids were harvested as described in the legend to Fig. 1, and their yield was assessed by electron microscopy of negatively stained preparations as described in Table 1.

[b] Polyethylene glycol 6000.

[c] In control experiments, 10 mM cycloheximide was found to reduce total protein synthesis (as judged by [^{35}S]methionine incorporation into acid-insoluble material) by >97% in both Sf9 and CV-1 cells.

[d] 75 μg each of DNase I and pancreatic RNase A per ml.

[e] The number of capsids observed was less than half the number seen in standard reactions.

Little or no capsid formation was observed when extracts were incubated at 4°C rather than 27°C (Table 2). A high concentration (100 mM) of dithiothreitol prevented capsid assembly entirely, while the yield was decreased when incubations were performed in the presence of 0.5% Triton X-100. Several other conditions tested, however, were found to be compatible with capsid formation. Capsids assembled normally, for example, in the presence of 50 mM EDTA, 10 mM cycloheximide, or DNase plus RNase. Polyethylene glycol was not required for capsid formation, but it stimulated the level of assembly, particularly in extracts where the protein concentration was low (less than 10 mg of protein per ml).

Immunological analyses were carried out to test the ability of capsids formed in the cell-free system to react with monoclonal antibodies specific for VP5. The tests involved mixing assembly products with VP5-specific or control antibodies under conditions in which specific antibodies are known to precipitate HSV-1 B capsids. The six extracts (25 μl each) were combined in the presence of 50 mM EDTA plus 3% polyethylene glycol and incubated to allow capsids to form as described above. Capsids were then collected by centrifugation through a sucrose cushion as described in the legend to Fig. 1, resuspended in 450 μl of TNE buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, pH 7.5), centrifuged briefly (16,000 × *g* for 1 min) to remove clumps, and divided into three equal (150-μl) aliquots. To each was added 5 μl of one of the following purified (by adsorption elution from protein A-Sepharose) monoclonal antibodies: 6F (0.5 mg/ml), 3G (0.5 mg/ml), or 5C9 (5 mg/ml). Precipitates were allowed to form by incubation overnight at 4°C, collected by centrifugation at 16,000 × *g* for 1 min, resuspended in 50 μl of TNE and prepared for electron microscopy by negative staining with 2% uranyl acetate as described previously (10, 21). Capsids were photographed at a magnification of ×3,300 (data not shown) and counted on electron microscope negatives. Capsids were observed in precipitates produced after the addition of monoclonal antibodies 6F and 3G which recognize VP5 and precipitate HSV-1 B capsids (13), but not with 5C9, a control antibody (specific for the M_1 protein of influenza virus [25]). The average numbers of capsids per microscope field, for example, were 37 (n = 5), 64 (n = 5), and 0 (n = 3) for precipitates produced by 6F, 3G,

and 5C9, respectively. We conclude that the capsids formed in the cell-free system are similar enough in structure to native B capsids that they can be recognized by the same VP5-specific antibodies.

The products of cell-free assembly were analyzed by sucrose density gradient ultracentrifugation, beginning with materials concentrated as described in the legend to Fig. 1. These were centrifuged on linear 20 to 50% sucrose gradients which, after centrifugation, revealed two bands as shown in Fig. 2a, lane 2. One (the bottom band) was found to sediment at the same rate as HSV-1 B capsids, while the other (top band) migrated slightly more slowly. Electron microscopic examination of material recovered from the bottom band showed that it consists almost entirely of closed structures with the dimensions of HSV-1 capsids (data not shown). Closed, apparently normal capsids were also found in the top band, but it contained, in addition, a substantial proportion of related structures such as unclosed capsids, forms resembling the "aberrant capsids" described by Thomsen et al. (22), and arcs, hemispheres, and other incomplete shells. The unfinished capsids appeared to have resulted from incomplete capsid formation or perhaps from damage to mature capsids.

Protein analysis by SDS-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) was carried out with the band of intact capsids (Fig. 2a, bottom band) resolved by sucrose gradient centrifugation. As shown in Fig. 2b (lane 3), VP5 was the predominant protein present, but VP19, VP21 (between VP19 and VP22a), VP22a, and VP23 were also observed. The ratio of VP19 to VP23 was comparable to that found in native B capsids (compare Fig. 2b, lanes 3 and 1). Although capsids assembled in the cell-free system contained VP22a, the amount present was significantly less than that seen in B capsids (compare Fig. 2b, lanes 3 and 1). Like its counterpart in B capsids, the VP22a found in cell-free capsids migrated as two closely spaced bands during SDS-polyacrylamide gel electrophoresis. No detectable VP24 or VP26 was observed in capsids formed in the cell-free system (data not shown). The protein composition of material in the top (incomplete capsid) band was similar to that of intact capsids (bottom band) except that the amount of VP19 was lower in top-band material (compare Fig. 2b, lanes 2 and 3).

The strong morphological resemblance of capsids formed in the cell-free system to authentic HSV-1 capsids indicates that cell-free assembly must be a reasonably faithful representation of capsid formation as it occurs in HSV-1-infected cells. The same conclusion is suggested by the similarity in protein composition between cell-free and in vivo capsids. The most important differences in protein composition between the two are (i) the lower content of scaffolding protein (VP22a) found in cell-free than in control B capsids and (ii) the absence of VP26 in capsids assembled in the cell-free system. The former result would be explained if the larger amount of VP22a present in native B capsids were greater than that minimally required for capsid formation or if a greater amount of VP22a is employed during cell-free assembly but most of it is lost after capsid formation is complete. The ability to form morphologically normal capsids in the absence of VP26 is consistent with previous studies of this protein. Thomsen et al. (22) observed that morphologically normal HSV-1 capsids form without VP26 in Sf9 cells multiply infected with recombinant baculoviruses. Further, HSV-1 capsids are found to retain their structural integrity when VP26 is removed by the extraction of B capsids with 2.0 M guanidine hydrochloride (4, 9). The absence of VP26 from cell-free capsids may result from the highly basic nature of this protein (pI = 11.6), which may result in its association with DNA when cells are lysed.

The conditions of capsid assembly described here were designed to require that assembly take place in the absence of living cells. Extracts were prepared by a method, multiple cycles of freezing and thawing, expected to lyse Sf9 cells; extracts were then centrifuged under conditions that would remove living cells if they were present. No intact cells were observed by microscopic examination of assembly-competent extracts. Further, capsid assembly was unaffected by the presence of cycloheximide at a concentration (10 mM) found to inhibit protein synthesis in Sf9 cells (Table 2). It is most probable, therefore, that capsid assembly in the extracts described here involves association of HSV-1 proteins in a cell-free environment. The higher level of capsid formation at 27°C than at 4°C raises the possibility that an enzymatic step may be required for capsid assembly. The maturational protease is not a candidate for such an enzymatic activity, however, since capsids are known to form in vivo in the absence of protease function (15, 22).

Several features of capsid assembly in the cell-free environment described here are similar to those observed during assembly in Sf9 cells multiply infected with recombinant baculoviruses as reported earlier (22). In both cases, the appearance of morphologically normal capsids depends on the presence of a source of VP5, VP19, VP22a, and VP23, while VP26 appears to be dispensable. Incomplete or aberrant capsids with similar morphologies were formed (in addition to authentic capsids) in both systems. It is most probable, therefore, that the pathways of capsid assembly are the same or very similar in the cell-free and baculovirus-infected Sf9 cell systems.

Inhibition of capsid assembly by dithiothreitol is of interest because it suggests that disulfide bond formation may be an obligatory step in the overall process. Zweig et al. (27) have documented the existence (in HSV-2) of a disulfide bond linking VP5 and VP19, and its formation may be the step affected by dithiothreitol. An effect on scaffold formation is suggested by recent observations showing that formation of toroidal scaffolds by purified VP22a (9) is strongly inhibited by dithiothreitol (11).

The availability of a cell-free system that supports HSV-1 capsid assembly, as described here, should facilitate studies of the assembly pathway and the identification of intermediates in capsid formation. In a cell-free system it is possible to manipulate the conditions of assembly such as the pH, ionic strength, or proportions of reaction components in ways that are difficult or impossible with live cells. Assembly intermediates can be trapped and identified directly without the need to lyse cells. Similarly, it should also be possible to employ the system described here to evaluate the effectiveness of candidate anti-HSV-1 therapeutic agents designed to disrupt capsid assembly. Test drugs can be added directly to reaction components without the need for them to enter living cells. Finally, the basic methods employed to create the cell-free system (i.e., production of recombinant baculoviruses and use of extracts prepared from infected Sf9 cells) should be readily adaptable for use with other herpesviruses, such as cytomegalovirus, Epstein-Barr virus, and varicella-zoster virus, in which genes analogous to those coding for HSV-1 capsid proteins have already been identified.

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