pH-dependent Fusion between the Flavivirus West Nile and Liposomal Model Membranes

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SUMMARY

Fusion between purified [3H]uridine-labelled West Nile virus (WNV) particles and liposomes containing RNase, was assayed by degradation of the viral RNA to trichloroacetic acid-soluble material. Fusion of virus with liposomes containing phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and cholesterol (at a molar ratio of 1:1:1:1.5) was found to be dependent on pH with maximum fusion occurring at pH 6.7 and below. At pH 6.6 fusion was rapid and was essentially complete within 2 min at 37 °C. At this time, approximately 50% of the viral RNA had been degraded and increasing the concentration of liposomes or time allowed for fusion increased this percentage only slightly. Fusion was dependent on temperature, was almost totally non-leaky and was not dependent on the presence of divalent cations. The lipid composition of liposomes was found to influence both the pH optimum for fusion and the maximum degree of fusion observed. Electron microscopy was used to visualize the fusion reaction between liposomes and virus particles.

INTRODUCTION

The ability of enveloped viruses to fuse with cellular or artificial lipid target membranes has been demonstrated for several viral families using a variety of different assay systems. Fusion can occur either at neutral pH (Paramyxoviridae, Herpesviridae), or be triggered by mildly acidic pH (Togaviridae, Rhabdoviridae and Bunyaviridae). Fusion has been proposed as being involved in the infectious entry process of enveloped viruses (for review, see Marsh, 1984), whereby viral genetic material is liberated into the cytosol either at the plasma membrane or in acidic prelysosomal endosomal compartments (Helenius et al., 1983).

Systems that have been used to study viral fusion include direct electron microscopic visualization of the fusion process (Mieselman et al., 1967; White et al., 1980; Matlin et al., 1981); fusion of cells ‘from without’ mediated by externally added virus particles (Meiselman et al., 1967; Väänänen & Kääriäinen, 1980; Maeda & Ohnishi, 1980; Huang et al., 1981; White et al., 1981; Gonzalez-Scarano et al., 1984); fusion of cells ‘from within’ via viral proteins expressed on the cell surface either because of viral infection (Huang et al., 1981; Mann et al., 1983; Gonzalez-Scarano et al., 1985) or because of infection with recombinant simian virus 40 (SV40), or microinjection of SV40-derived expression vectors containing cloned copies of genes coding for viral membrane glycoproteins (White et al., 1982a; Kondor-Koch et al., 1983; Riedel et al., 1984); haemolysis (Väänänen and Kääriäinen, 1979; Maeda & Ohnishi, 1980; Huang et al., 1981); and finally fusion with artificial liposomal vesicles (Haywood, 1974; White & Helenius, 1980; White et al., 1982b; Hsu et al., 1983).

No evidence exists, however, that viruses belonging to the family Flaviviridae (Westaway et al., 1985) can fuse with membranes. Thus the present study was carried out to examine the fusion activity of West Nile virus (WNV) with artificial liposomal model membranes at various pH values using a system similar to that used by White & Helenius (1980).
METHODS

Cells. Vero cells were maintained as described previously (Gollins & Porterfield, 1984).

\([^3]H\)Uridine-labelled virus. Subconfluent monolayers of Vero cells in 75 cm\(^2\) flasks were infected at 50 p.f.u./cell with a suckling mouse brain suspension of WNV for 1 h at 37 °C. The cells were then washed once and maintained in normal growth medium with 10 h after infection. Cells were then washed twice with Dulbecco's MEM (DMEM) containing 0.2% bovine serum albumin (BSA) and then 5 ml of radioactive labelling medium was added per flask. This consisted of DMEM buffered with 10 mM-EPSS and 10 mM-HEPES (pH 7.8), containing 0.2% BSA, 1.5 μg per ml actinomycin D and 0.5 mCi per flask of \([^3]H\)uridine (Amersham, sp. act. 50 Ci/mmol). At 32 h after infection the tissue culture supernatants were harvested and pooled. These were clarified by centrifugation at 10000 g for 30 min and virus was then concentrated and purified by velocity sedimentation on sucrose gradients as described by Westaway & Reedman (1969). Virus particles were stored at −80 °C in 0.14 M-NaCl, 0.01 M-Tris-HCl pH 8.0 containing 0.1% BSA and approximately 15% (w/v) sucrose. In the stocks of purified WNV particles used there were approximately 10\(^{10}\) virus particles per ml (from latex particle counts), and 1.43 × 10\(^7\) c.p.m. per ml.

Unlabelled virus preparation. Unlabelled WNV was prepared as described in Gollins & Porterfield (1985) and further purified by equilibrium density centrifugation on a 20 to 50% (w/w) sucrose gradient in 0.14 M-NaCl, 0.01 M-Tris-HCl pH 8.0 containing 0.1% BSA and approximately 15% (w/v) sucrose. In the stocks of purified WNV particles used there were approximately 10\(^{10}\) virus particles per ml.

Preparation of liposomes and the basic fusion assay. 1.25 μmol each of phosphatidylethanolamine (PE) from soybean (type IV), phosphatidylcholine (PC) from egg yolk (type V-E), sphingomyelin (Sph) from bovine brain and 1.875 μmol of cholesterol (Cho) were combined in 1.0 ml of chloroform:methanol (2:1). All lipids were from Sigma and of the highest purity available. The mixture was evaporated onto the sides of a glass tube at room temperature using a rotary evaporator and then evaporated under vacuum for an additional 90 min. One ml of MES-HEPES buffer [0.13 M-NaCl, 10 mM-HEPES, 10 mM-MES pH 7.2, 0.2% lipid-free BSA (Sigma)] was then added containing RNase (type III-A from bovine pancreas; Sigma) at 2 mg/ml. A few 1 mm diameter glass beads (Sigma) were then added and the mixture was vortexed vigorously for 5 min. The mixture was then subjected to sonication for 5 min more in an 'Electrosonic' water bath sonicator (Headland, London) and the resultant suspension of liposomes was diluted 1 in 5.5 in MES-HEPES buffer and then chilled to 0 °C on ice. The final concentration of RNase inside liposomes was therefore assumed to be 2 mg per ml and outside liposomes was 0.36 mg per ml. The amount of degradation of viral RNA to TCA-soluble material occurring in the absence of RNase was therefore approx. 2 mg per ml and outside liposomes was 0.36 mg per ml.

The tubes were immediately immersed in a 37 °C water bath for 2 min and the contents of each tube were then precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) in distilled water. The tubes were left on ice for 30 min and then centrifuged for 5 min in a microfuge and an aliquot of the supernatant was counted in 'Unisolve E' scintillation fluid (Koch-Light) in a Packard Tri-Carb liquid scintillation counter to give a value for TCA-soluble radioactivity. The basic assay was varied in individual experiments as described in Results. All results presented are the average of three or more experiments, and the results did not differ by more than 10% between different experiments.

Assay of virus attachment to liposomes. This was carried out essentially as described by Mooney et al. (1975). \([^3]H\)Uridine-labelled WNV was incubated in 600 μl of MES-HEPES buffer (pH 6.6) for 10 min at 37 °C with or without liposomes (that did not contain RNase). The mixture was cooled to 0 °C, brought to pH 7.6 with dilute NaOH, and then layered over a discontinuous sucrose gradient consisting of 3.8 ml of 15% (w/v) sucrose on top of a 0.7 ml cushion of 70% (w/v) sucrose in MES-HEPES buffer (pH 7.6). The tubes were then centrifuged for 1.5 h at 10000 g for 30 min and virus was then concentrated and purified by velocity sedimentation on sucrose gradients as described by Westaway & Reedman (1969). Virus particles were stored at −80 °C in 0.14 M-NaCl, 0.01 M-Tris-HCl pH 8.0 containing 0.1% BSA and approximately 15% (w/v) sucrose. In the stocks of purified WNV particles used there were approximately 10\(^{10}\) virus particles per ml.

RESULTS

Basic fusion assay and pH dependence of WNV membrane fusion with liposomes (Fig. 1)

The amount of degradation of viral RNA to TCA-soluble material occurring in the absence of liposomes was found to be very low in the presence of RNase both at 2 mg/ml and 0.36 mg/ml,
Fig. 1. The pH dependence of WNV fusion with liposomes. The degradation of [3H]uridine-labelled WNV RNA to TCA-soluble material at various pH values was assayed as described in Methods. The assay was for 2 min at 37 °C and liposomes contained PE, PC, Sph and Cho at a ratio of 1:1:1:1.5 (final concentration of lipid in all experiments 1 mM). Assay conditions used were as follows. Liposomes loaded with 2 mg/ml RNase with 0.36 mg/ml RNase on their outside (○) or presence (□) of 1% Triton X-100. Liposomes with RNase solely on their outside at 2 mg/ml (■) or 0.36 mg/ml (△). Liposomes absent but RNase present at 2 mg/ml (▲) or 0.36 mg/ml (▲). A total of 2650 c.p.m. was precipitated with TCA for each point plotted. When liposomes were included but RNase was totally absent, or when both liposomes and RNase were absent, approximately 25 c.p.m. was not precipitable with TCA, regardless of pH, and this amount has been subtracted from all values shown.

Fig. 2. The time dependence of WNV fusion with liposomes. The degradation of [3H]uridine-labelled WNV RNA to TCA-soluble material at various pH values was assayed as described in Methods. The assay was for various times at 37 °C and liposomes contained PE, PC, Sph and Cho at a ratio of 1:1:1:1:5 (final concentration of lipid 1 mM). Assay conditions were as follows. Liposomes loaded with 2 mg/ml RNase with 0.36 mg/ml RNase on their outside at pH 6.6 (■), 7.1 (▲) or 8.0 (□). A total of 2670 c.p.m. was precipitated with TCA for each point plotted.

indicating that the structure of the intact enveloped virus was effective in protecting the viral RNA from attack by nuclease. However, when liposomes loaded with RNase were included in the mixture, an increasing amount of degradation of viral RNA was seen between pH 8.0 and 6.7, with maximal degradation at pH 6.7 or below. When the small amount of background degradation by RNase in the absence of liposomes was taken into account it was found that the increase in degradation occurring between pH 8.0 and 6.7 was approximately ninefold and between pH 7.1 and 6.7 was approximately 2.8-fold.

The use of mixtures in which Triton X-100 was included demonstrated that when the lipid membrane was removed from virus particles, more than 90% of the viral RNA was susceptible to hydrolysis by the nuclease at all pH values under the assay conditions used.

However, a maximum of only approximately 50% of the input viral RNA could be degraded when uncoating took place through interaction with liposomes. This percentage was increased only slightly when the liposomal lipid concentration was increased to 3 mM (not shown), or when the incubation was carried out for up to 15 min at 37 °C (Fig. 2), rather than the 2 min used in the basic assay.
When incubations were carried out with RNase solely on the outside of liposomes only a small amount of WNV RNA was susceptible to degradation. This indicated that the majority of the degradation of viral RNA seen with liposomes containing RNase in the basic fusion assay protocol (with an assumed 2 mg/ml RNase inside liposomes and 0.36 mg/ml outside them) was most probably due to 'non-leaky' fusion between liposomal and viral membranes resulting in mixing of viral and liposomal contents. The viral RNA degradation on incubation with liposomes solely with RNase on their outside, is most probably due to a small amount of 'leaky fusion' (White et al., 1982b), allowing access of external RNase to the viral RNA.

**Effect of time on fusion (Fig. 2)**

Time course experiments showed that the fusion reaction occurred very rapidly and was essentially complete by 2 min after warming to 37 °C at pH 6.6. Control experiments without liposomes showed that there was a small increase in the background degradation of RNA within whole virus particles between 2 and 15 min.

**Effect of temperature on fusion (Fig. 3)**

At pH 6.6 fusion was absolutely dependent on temperature; no fusion was seen at 0 °C, and the magnitude of fusion increased progressively between 0 °C and 37 °C (Fig. 3). Controls that included Triton X-100 demonstrated that the temperature dependence of degradation at pH 6.6 was not due to differing activities of the RNase at different temperatures. The reduction in degradation seen when all the virus RNA was uncoated with detergent, was approximately one-third between 55 °C and 0 °C. This could not account for the absolute reduction in degradation seen in the absence of detergent at pH 6.6 between these temperatures.

In contrast, at pH 8.0 little fusion was seen between 0 °C and 37 °C although at 45 and 55 °C fusion occurred almost to the extent seen at pH 6.6. Control experiments carried out in the presence of RNase (0.36 or 2.0 mg/ml) but in the absence of liposomes showed a relatively small increase in degradation between 37 and 45 °C at pH 8.0 (not shown). This implied that the large rise in degradation seen between 37 and 45 °C in the presence of liposomes was probably due to increased fusion at the higher temperature and that constraints on WNV fusion at pH 8.0 at physiological temperature (37 °C) break down when the temperature is elevated.

**Effect of lipid concentration on fusion (Fig. 4)**

At low concentrations of lipid (0.001 to 0.01 mM) the extent of fusion was dependent on the lipid concentration. At higher concentrations of lipid (0.1 to 1.0 mM) the lipid concentration was almost non-limiting with regard to the extent of fusion.

**Divalent cations and fusion**

When EDTA up to 5 mM was included in the fusion mixture no decrease in the extent of fusion was observed at pH 6.6, indicating that divalent cations were not needed for the fusion reaction (not shown).

**Effect of liposomal lipid composition on fusion (Fig. 5 and 6)**

Fusion of virions with liposomes composed of PE, PC, Sph and Cho (standard liposomes) was not totally dependent on any one component of the liposomes (Fig. 5). Elimination of PE, PC or Cho produced reductions of approximately a quarter to a third in comparison to the maximum level of fusion observed with standard liposomes. Elimination of Sph produced a small rise in the maximum level of fusion seen.

However, if the details of the fusion profiles were examined it was seen that elimination of Cho totally reduced the fusion seen between pH 8.0 and 7.1 compared to standard liposomes. Elimination of Sph partially reduced the fusion seen between pH 8.0 and 7.1 when compared to standard liposomes (Fig. 5). Furthermore, elimination of Cho consistently shifted
the highest pH at which maximum fusion was seen, to a more acidic pH. With Cho-containing liposomes maximum fusion was seen at approximately pH 6.7 whereas little fusion was seen at this pH in the absence of Cho, the maximum being reached at approximately pH 6.3 (Fig. 5).

When more simple liposomes consisting of PC alone were examined (Fig. 6) it was found that they were totally fusion-inactive. However, if Cho was included in their composition (i.e. PC + Cho), then the maximum level of fusion was comparable to that obtained with standard liposomes (consisting of PC + PE + Cho + Sph). PC + Sph liposomes did not support fusion and PC + PE liposomes showed only a small amount of fusion activity at lower pH values (Fig. 6).
Fig. 5. The dependence of WNV fusion with liposomes on the liposomal lipid composition. The degradation of [3H]uridine-labelled WNV RNA to TCA-soluble material at various pH values was assayed as described in Methods. The assay was for 2 min at 37°C and liposomes were of various compositions (final concentration of lipid in all cases 1 mM). Assay conditions were as follows. Liposomes containing PE, PC, Sph and Cho (ratio 1:1:1:1.5) (○); liposomes containing PE, PC and Cho (ratio 1:1:1) (▲); liposomes containing PE, Sph and Cho (ratio 1:1:1) (△); liposomes containing PC, Sph and Cho (ratio 1:1:1) (■); liposomes containing PE, PC and Sph (ratio 1:1:1) (●). A total of 1900 c.p.m. was precipitated with TCA for each point plotted.

Fig. 6. The dependence of WNV fusion with liposomes on the liposomal lipid composition. This was carried out as for Fig. 5. Liposomes containing PE, PC, Sph and Cho (ratio 1:1:1:1.5) (○); liposomes containing PC, Sph and PE (ratio 2:1) (□); liposomes containing PC and Sph (ratio 2:1) (△); liposomes containing PC and Cho (ratio 2:1) (▲). A total of 1900 c.p.m. was precipitated with TCA for each point plotted.

Electron microscopic evidence for fusion between viral and liposomal membranes (Fig. 7)

In the absence of liposomes at pH 6.4 virus particles appeared to have a surface structure composed of fine spikes and a small degree of aggregation of virus particles was seen (Fig. 7a).

In the absence of liposomes at pH 8.0 the appearance of virus particles was very similar to that seen at pH 6.4 and very little aggregation was seen (Fig. 7b and c).

When liposomes were present these were seen to consist of a heterogeneous population of multilamellar and unilamellar lipid vesicles. At pH 6.4 many virus particles could be seen with their membranes in intimate contact with liposomal membranes and many images were seen which suggested that substantial fusion had occurred between viral and liposomal membranes. In cases where stain had not penetrated the outer liposomal membrane, discrete areas of membrane were sometimes visible covered with viral spikes in various degrees of organization, presumably just after a fusion event had taken place (Fig. 7d and i). Where stain had penetrated...
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Fig. 7. Electron microscopic evidence for fusion of WNV with liposomes. Electron microscopy was carried out as in Methods. (a) Virus particles incubated at pH 6.4 in the absence of liposomes. (b, c) Virus particles incubated at pH 8.0 in the absence of liposomes. (d to i) Virus particles incubated at pH 6.4 in the presence of liposomes. (j, k) Virus particles incubated at pH 8.0 in the presence of liposomes. Arrowheads show whole virus particles, small arrows viral spikes and large arrows show viral cores. L. liposomes. Bar marker represents 100 nm. The 'pits' seen in liposomes in (g) occurred occasionally both in experimental samples with virus and in control samples in the absence of virus. They represent small areas of dried negative stain trapped on top of liposomes due to the staining procedure and have no relevance to the fusion process.
the outer liposomal membrane, viral cores were sometimes visible and were recognizable by
their smaller diameter (approximately 33 nm) when compared to whole virus particles
(approximately 45 nm) (Fig. 7g and i). In certain cases cores appeared to be trapped between
the lipid bilayers of multilamellar liposomes after a fusion event had occurred between the viral
envelope and the outermost liposomal membrane (Fig. 7f and i). Viral spikes were sometimes
visible immediately surrounding the cores (Fig. 7f, g, h and i), or appeared to be in the process of
diffusing along the outer liposomal membrane away from the immediate fusion area (Fig. 7e
and g).

At pH 8.0 some virus particles were seen bound to liposomes, though in far smaller numbers
than at pH 6.4 and little evidence for fusion was seen (Fig. 7j and k).

**DISCUSSION**

The main points emerging from the results presented in this paper are that WNV, a member
of the family Flaviviridae, can fuse with artificial lipid membranes in a pH-dependent manner,
and that the lipid composition of the target membrane affects both the extent of fusion and the
pH optimum for fusion.

It is possible that the pH optimum demonstrated for fusion of WNV with liposomes is
substantially different from that for fusion of WNV with cell membranes. In a recent
morphological study of the entry pathway of WNV into the murine macrophage-like cell line
P388D1 (Gollins & Porterfield, 1985), no evidence was found for fusion of WNV with the cell
surface membrane at pH 7.4 when viral antigens were identified by indirect labelling with gold-
conjugated antibody.

Recently it has been found that several different flaviviruses can cause haemolysis of 24 h
chick erythrocytes with a pH optimum of 5.4, with no haemolysis demonstrable above pH 6.0
(Cammack & Gould, 1985). It seems likely that haemolysis is indicative of a fusion reaction
occurring between viral and red blood cell membranes because of the coincidence of the
optimum pH for haemolysis with the pH at which several viruses can fuse with red blood cells
(Väänänen & Kääriäinen, 1980; Maeda & Ohnishi, 1980). Thus it would appear that the pH
optimum for flavivirus fusion with red blood cell membranes is very different from that for
fusion with liposomes (i.e. pH 5.4 compared to pH 6.7).

A less extreme example of the composition of the target membrane being able to affect the pH
optimum for fusion is also seen in the present study. With liposomes containing PE, PC, Sph and
Cho, the highest pH at which maximum fusion was seen was pH 6.7. When Cho was omitted
from the liposome composition, however, the maximum fusion pH was shifted 0.4 pH units to
pH 6.3 (Fig. 5).

Our inference from the above observations is that at least in the case of flaviviruses, both the
composition of the target membrane (conceivably both lipids and proteins), as well as possible,
as yet undefined, pH-dependent conformational changes occurring in the virus particles
themselves, by analogy with some other enveloped viruses (White et al., 1983), might be
responsible for determining the exact pH values at which fusion takes place. Similarly a recent
study (Huang et al., 1985) has indicated a role for the target membrane in dictating the exact pH
optimum for fusion of influenza virus with red blood cells.

In addition to the optimum pH for fusion being dependent on the membrane lipid
composition, the magnitude of the fusion seen also depends on membrane lipid composition. In
simple liposomes containing PC only, PC + PE, or PC + Sph, virtually no fusion occurred (Fig.
6); however, liposomes containing PC + Cho showed fusion comparable with that of standard
liposomes. This might be due, at least partially, to the potential fusion-promoting properties of
Cho, which include lowering the hydration of lipid vesicles at molar fractions higher than 0.22
(Newman & Huang, 1975), decreasing water penetration into the bilayer (Simon et al., 1982) and
inducing phase separations in Cho-phospholipid membranes (Tajima & Gershfeld, 1978; Lentz
et al., 1980).

The presence of Sph and PE together (though neither Sph nor PE alone, to any great extent) in
PC liposomes could partially compensate for the absence of Cho in terms of fusion (Fig. 5 and 6).
Potential fusion-promoting properties of PE include the fact that PE is less hydrated than other
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phospholipids (Rand, 1981), and has a strong tendency to form hexagonal II-phase structures in membranes (Cullis & de Kruijff, 1979).

The ability to fuse with membranes at mild acidic pH probably has a role in the flavivirus infectious cellular entry process. Lysosomotropic amines can inhibit flavivirus infection of cells (Gollins & Porterfield, 1984; Brandriss & Schlesinger, 1984), and inhibit penetration of virus cores into the cytosol from endosomal prelysosomal compartments (Gollins & Porterfield, 1985), probably by raising the endosomal pH above a level at which viral–endosomal membrane fusion can occur. The high concentrations of ammonium chloride (50 mM) previously found necessary to block WNV infection of P388D1 cells at neutral pH (Gollins & Porterfield, 1984) possibly suggests that the intraendosomal pH threshold for fusion is above the threshold (pH 6-2) for the fusion with cell membranes of Semliki Forest virus (White et al., 1980) and vesicular stomatitis virus (Matlin et al., 1982). In agreement with this, recent unpublished results in our laboratory using [3H]uridine-labelled WNV in an RNase degradation assay similar in principle to the liposomal uncoating assay used in this paper, have shown that if WNV is prebound to the P388D1 plasma membrane at 0 °C, then treated for short time periods at various pH values at 37 °C, uncoating occurs with a threshold of approximately pH 6-6.

In the present study the amount of virus associated with liposomes was measured by separating unbound from bound virus particles on the basis of density as described in Methods. It was found that on incubation with liposomes for 10 min at 37 °C, pH 6-6, more than 85% of virus particles were associated with liposomes (not shown), whereas under the same conditions approximately 55% of total viral RNA had been specifically degraded by RNase contained in liposomes. Thus there was a definite proportion of liposome-associated viral RNA that was not susceptible to degradation. Whether this represented virus particles that were merely bound but not fused, or whether virus particles had fused but their RNA was not totally susceptible to degradation by intraliposomal RNase, is unclear at the present time. If the latter alternative is true, then this would mean that viral uncoating via fusion with a lipid bilayer leaves the viral RNA in a more protected conformation than when the viral membrane is removed by detergent (when more than 90% of viral RNA is susceptible to degradation with RNase, Fig. 1).

It is possible that factors that are present in the cell, in addition to low endosomal pH, are necessary for complete uncoating of WNV RNA to take place. For example, Wilson (1984a, b) has recently shown that eukaryotic ribosomes can bring about the uncoating of the tobacco mosaic virus capsid in cell-free systems, i.e. co-translational uncoating. One can speculate that, by analogy, it is possible that in the cell factors involved in the subsequent translation of WNV positive single-stranded RNA might be necessary for full infectious nucleocapsid disassembly, after the initial pH-dependent viral–endosomal fusion event has taken place. With neither alphaviruses nor orthomyxoviruses have pH-dependent liposomal uncoating systems been shown to result in transcriptionally or translationally active uncoated viral RNA, and clearly future studies will have to examine this aspect of pH-dependent enveloped virus fusion-mediated uncoating in order that potential additional factors involved in full infectious nucleocapsid disassembly might be identified.

REFERENCES


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