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# Estimation by Radiation Inactivation of the Size of Functional Units Governing Sendai and Influenza Virus Fusion†

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ABSTRACT: The target sizes associated with fusion and hemolysis carried out by Sendai virus envelope glycoproteins were determined by radiation inactivation analysis. The target size for influenza virus mediated fusion with erythrocyte ghosts at pH 5.0 was also determined for comparison; a value of 57 ± 15 kDa was found, indistinguishable from that reported previously for influenza-mediated fusion of cardiolipin liposomes [Gibson, S., Jung, C. Y., Takahashi, M., & Lenard, J. (1986) *Biochemistry 25*, 6264–6268]. Sendai-mediated fusion with erythrocyte ghosts at pH 7.0 was likewise inactivated exponentially with increasing radiation dose, yielding a target size of 60 ± 6 kDa, a value consistent with the molecular weight of a single F-protein molecule. The inactivation curve for Sendai-mediated fusion with cardiolipin liposomes at pH 7.0, however, was more complex. Assuming a "multiple target–single hit" model, the target consisted of 2–3 units of ca. 60 kDa each. A similar target was seen if the liposomes contained 10% gangliosides or if the reaction was measured at pH 5.0, suggesting that fusion occurred by the same mechanism at high and low pH. A target size of 261 ± 48 kDa was found for Sendai-induced hemolysis, in contrast with influenza, which had a more complex target size for this activity (Gibson et al., 1986). Sendai virus fusion thus occurs by different mechanisms depending upon the nature of the target membrane, since it is mediated by different functional units. Hemolysis is mediated by a functional unit different from that associated with erythrocyte ghost fusion or with cardiolipin liposome fusion.

Radiation analysis has been used previously to determine the inactivation target size for several functions of the influenza virus membrane (Gibson et al., 1986). Fusion of influenza with cardiolipin (CL)[1] liposomes was exponentially inactivated as a function of increasing radiation dose, corresponding to a radiation target size of 53–55 kDa, in reasonable agreement with the molecular weight of a single molecule of the influenza fusion protein HA. On the other hand, the targets governing

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[1] Abbreviations: CL, cardiolipin; $R_{18}$, octadecylrhodamine B chloride; RET, resonance energy transfer; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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inactivation of leakage—either of entrapped dye from CL liposomes or of hemoglobin from erythrocytes (hemolysis)—were larger and more complex than those for fusion. The inactivation curves using both leakage assays showed a pronounced shoulder, followed by a steep drop, suggesting as the simplest possibility the involvement of several entities of 300–400 kDa in the functional unit governing leakage (Gibson et al., 1986).

The fusion properties of Sendai virus, like those of influenza, have been intensively studied. Several studies have indicated that Sendai fuses best at neutral and slightly alkaline pH values, unlike influenza, which requires a pH of ca. 5.5 or below. The Sendai fusion protein, F ($M_r$ ca. 65 000), has some sequence homology with the influenza fusion protein HA and resembles it also in requiring a specific proteolytic cleavage for activation. A second Sendai virus glycoprotein, HN ($M_r$ ca. 67 000), possesses both the hemagglutinating and neuraminidase activities of the virus [review by White et al. (1983)]. Some reports have claimed that HN must be present along with F for fusion to occur (Miura et al., 1982; Nakanishi et al., 1982; Nussbaum et al., 1984; Ozawa et al., 1979), while others find that F alone is sufficient for fusion (Hsu et al., 1979; Markwell et al., 1985). In contrast with influenza, for which the three-dimensional structure of both glycoproteins is known, not even the subunit composition of a spike has yet been reliably determined for either Sendai glycoprotein.

Recent reports have suggested that fusion of both Sendai and influenza with CL and other acidic liposomes occurs via a mechanism different from that of fusion with biological membranes such as erythrocyte ghosts (Citovsky et al., 1985; Amselem et al., 1986; Klappe et al., 1986; Stegmann et al., 1986). This conclusion was based on differential inactivation of the two activities by a variety of agents and on marked differences in pH dependence. Fusion of Sendai with CL liposomes was found to be markedly greater at pH 5.0 than at pH 7.0 (Amselem et al., 1986; Klappe et al., 1986), an observation that we have confirmed (unpublished observations). In this paper we show by radiation inactivation analysis that the radiation target size for Sendai–erythrocyte ghost fusion is substantially different from that for Sendai–CL liposome fusion. This confirms that the two fusion reactions must occur by different mechanisms. Sendai-induced hemolysis, which requires leakage of hemoglobin from the erythrocyte membrane following fusion, is governed by a functional unit of yet another size.

## Materials and Methods

*Virus.* Influenza strain APR/8/34 and Sendai strain Z were grown in 10–11-day-old embryonated eggs and purified on 5–40% potassium tartrate gradients.

*Chemicals.* Cardiolipin, the sodium salt from bovine heart (CL), was purchased from Sigma Chemical Co. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (N-NBD-PE) and octadecylrhodamine B chloride ($R_{18}$) were obtained from Molecular Probes, Inc. *N*-(Lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (N-Rh-PE) was from Avanti Polar Lipids, Inc. Brain gangliosides (lyophilized Folch extract) were obtained from Supelco. All other chemicals were of the highest purity available.

*Radiation Inactivation.* This was carried out by Dr. Chan Y. Jung exactly as described previously (Gibson et al., 1986).

*Target-Size Analysis.* In those cases where radiation inactivation followed the exponential relationship predicted by a single hit–single target model (Kempner & Schlegel, 1979; Jung, 1984), the empirical relationship of Kepner and Macey (1968), $M_r = (6.4 \times 10^5)/D_{37}$, was used, where $D_{37}$ is the dose in megarads resulting in 37% residual activity and $M_r$ is the molecular mass of the target in daltons. Data were fitted by using unweighted least-squares regression analysis. The best-fit line was not arbitrarily constrained to pass through the 100% activity–zero dosage intercept. All values shown are averages of triplicate determinations. Each inactivation curve was constructed from values obtained from two or three separate irradiation experiments.

*Hemolysis.* Hemolysis of human red blood cells by Sendai was performed in 140 mM NaCl, 10 mM sodium citrate, 10 mM Hepes, and 0.1 mM EDTA, pH 7.0, by using the technique previously described (Lenard & Miller, 1981).

*Erythrocyte Ghosts.* Human erythrocyte ghosts were prepared from outdated blood by the procedure of Steck and Kant (1974).

*Preparation of $R_{18}$-Labeled Virions.* Influenza and Sendai virions were labeled with quenching concentrations of $R_{18}$ according to the method detailed by Hoekstra et al. (1984), with modifications described by Citovsky et al. (1985). For influenza, 5 μL of an ethanolic solution of $R_{18}$ (100 μg/mL) was injected into 500 μL of buffer (containing 36 μg of virus protein). The solution was incubated in the dark for 15 min at room temperature. The sample was then diluted to 10 mL with cold buffer and centrifuged for 30 min at 31 500 rpm at 4 °C. A small pink pellet could be seen at the bottom of the tube, and 9.35 mL of the supernatant was carefully removed. The pellet was then resuspended by vortexing into the remaining volume. The same procedure was followed for Sendai, except that 2.5 μL of an ethanolic solution of $R_{18}$ (25 μg/mL) was injected into 500 μL of buffer containing 42 μg of Sendai protein.

It was observed that the higher the radiation dose to which Sendai or influenza was exposed, the less incorporated $R_{18}$ was recovered following centrifugation. Gel filtration on a Sephadex G-75 column was therefore used to separate the labeled sample from unincorporated $R_{18}$ in one series of experiments. When purified in this way, both the total amount of virus-bound $R_{18}$ and the probe density as estimated by the degree of quenching of the $R_{18}$ fluophore were identical in all samples, regardless of radiation dose, suggesting that irradiation did not grossly perturb the arrangement of membrane lipids. Fusion results from samples that had been purified by gel filtration were essentially the same as those reported in this paper; the centrifugation method was routinely used for reasons of convenience.

*$R_{18}$ Fusion Assay.* To the $R_{18}$-labeled influenza or Sendai virus prepared as above (5 μg) were added erythrocyte ghosts in 10-fold excess, based on phospholipid content. The mixture was adjusted to pH 5.0 or 7.0 as indicated, in a total volume of 300 μL, by addition of buffer (140 mM NaCl, 10 mM sodium citrate, 5 mM Hepes, and 0.1 mM EDTA) at the appropriate pH. Samples were incubated on ice for 15 min and at 37 °C for an additional 45 min. Fusion was measured against a ghost-free blank as the increase in fluorescence at excitation and emission wavelengths of 560 and 590 nm, respectively. The 100% value, taken as the fluorescence measured after the addition of Triton X-100 (0.5% v/v), was determined separately for each labeled sample.

*Preparation of Liposomes.* N-NBD-PE and N-Rh-PE (1.0 mol % each) were incorporated into small unilamellar CL liposomes by ethanolic injection (Batzri & Korn, 1973) as described earlier (Gibson et al., 1986).

*RET Fusion Assay.* To monitor virus–CL liposome fusion, the RET method (Struck et al., 1981; Stegmann et al., 1985)

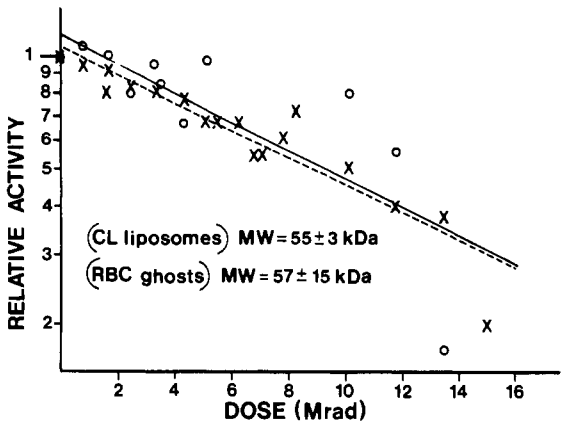


FIGURE 1: Radiation-induced loss of fusion activity from influenza virions: (O) fusion between $R_{18}$-labeled virions and erythrocyte ghosts, measured by relief of fluorescence quenching; (×) fusion between unlabeled virions and CL liposomes labeled with N-NBD-PE and N-Rh-PE, measured by RET [data from Gibson et al. (1986)]. Activity of the unirradiated sample (relative to the appropriate 100% value) was (O) 0.48 and (×) 0.80.

was used as previously described (Gibson et al., 1986). Samples were incubated for 30 min at 37 °C.

## Results

In a previous radiation inactivation study of influenza virus (Gibson et al., 1986), fusion was measured by using only CL liposomes. Inactivation of fusion activity as a function of increasing amounts of radiation occurred with simple exponential dependence, consistent with a target of ca. 55 kDa. Fusion of influenza virus with erythrocyte ghosts, which has recently been found to possess several characteristic differences from CL liposome fusion (Stegmann et al., 1986), was not studied.

The radiation inactivation characteristics of the influenza–erythrocyte ghost fusion system were therefore determined. As shown in Figure 1, a simple exponential inactivation was found, corresponding to a target size of 57 ± 15 kDa, indistinguishable from that previously determined with the influenza–CL liposome fusion system (Gibson et al., 1986). The target size for fusion with CL liposomes was normalized in the previous study to that of neuraminidase in the same samples to give a molecular mass of 68–70 kDa for influenza–CL liposome fusion. The target size for influenza–ghost fusion becomes 73 ± 19 kDa when the same normalization ratio is used. These numbers are very close to the molecular weight of a single HA molecule, which is ca. 75 000.

The radiation inactivation curves for Sendai–ghost fusion and for Sendai–CL liposome fusion are shown in Figure 2. The curve for Sendai–ghost fusion resembled that of influenza–ghost fusion (Figure 1) in having a simple exponential form. The target size corresponded to a molecular mass of 60 ± 6 kDa, close to that of a single F or HN molecule. The inactivation profile for Sendai–CL liposome fusion, on the other hand, possessed a pronounced shoulder with enhanced activity of over 150% of unirradiated controls at <14-Mrad irradiation (Figure 2). Clearly, Sendai–ghost fusion depends upon a different sized functional unit than Sendai–CL liposome fusion and must therefore occur by a different mechanism.

Radiation inactivation of Sendai–CL fusion was investigated under two other fusion conditions. The radiation dependence of inactivation at pH 7.0 in the presence of 10% (w/w) added ganglioside and at pH 5.0 in the absence of ganglioside is shown in Figure 3. Both conditions gave similar inactivation curves, showing a pronounced shoulder. As detailed below, the shapes of the inactivation curves in Figure 3 are similar

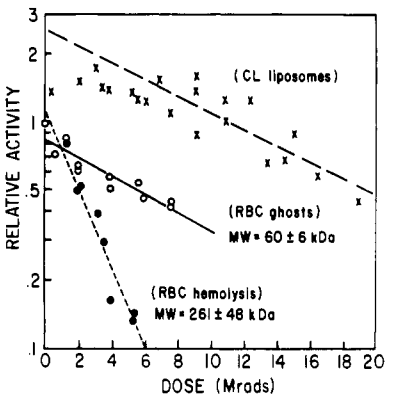


FIGURE 2: Radiation-induced loss of fusion and hemolytic activity from Sendai virions: (O) fusion between $R_{18}$-labeled virions and erythrocyte ghosts, measured by relief of fluorescence quenching; (×) fusion at pH 7.0 between unlabeled virions and CL liposomes labeled with N-NBD-PE and N-Rh-PE, measured by RET; (●) hemolysis. Activity of the unirradiated sample (relative to the appropriate 100% value) was (O) 0.50, (×) 0.32, and (●) 0.86.

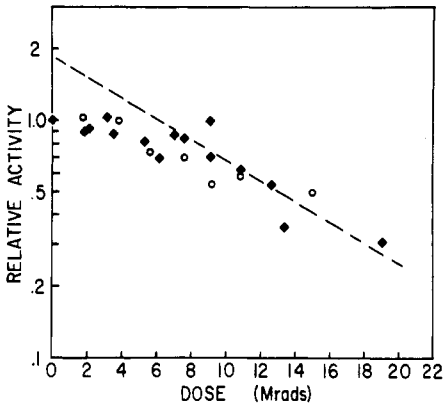


FIGURE 3: Radiation-induced loss of CL liposome fusion activity from Sendai virions. Sendai–CL liposome fusion was measured as in Figure 2 at pH 5.0 (◆) or at pH 7.0 with CL liposomes containing 10% (w/w) gangliosides (O, single irradiation experiment). Activity of the unirradiated sample (relative to the appropriate 100% value) was (◆) 0.62 and (O) 0.38.

to that for Sendai–CL fusion at pH 7.0 without gangliosides (Figure 2), except that they lack any enhancement of fusion activity at low radiation doses.

The radiation inactivation profile of Sendai-induced hemolysis is also shown in Figure 2. The points lie on a simple exponential curve corresponding to a target size of 261 ± 48 kDa, a value 3–4 times as large as that found for Sendai–ghost fusion. The shape of this curve is quite different from that previously found for influenza virus induced hemolysis, which possessed a pronounced shoulder (Gibson et al., 1986), and differs also from both fusion inactivation curves shown in Figure 2.

## Discussion

The present results indicate that in both Sendai and influenza viruses a single viral glycoprotein molecule comprises the functional unit governing fusion with erythrocyte ghosts. Since the entire molecule is likely to be destroyed by a single radiation event (Haigler et al., 1985; Kempner & Miller, 1983), the functional unit could in principle be only part of the molecule. A single glycoprotein molecule, however, appears to be sufficient to carry out a fusion event; aggregates or complexes of several molecules are not required. The molecular weights of F and HN molecules are nearly the same, so these results alone cannot distinguish which of the two viral

glycoproteins confers the activity. On the basis of many previous studies, however, it is clear that F is the major or sole protein mediating fusion of Sendai virus with biological membranes (White et al., 1983), so we may confidently assign the target for Sendai–ghost fusion (Figure 2) to a single molecule of F-protein. It should be noted that a target the size of a single glycoprotein molecule is incompatible with the proposal (Miura et al., 1982; Nakanishi et al., 1982; Nussbaum et al., 1984; Ozawa et al., 1979) that F and HN are both required for fusion with ghosts, since any such requirement would necessarily create a larger and/or more complex target for radiation inactivation.

Fusion of Sendai with CL liposomes, on the other hand, is governed by a functional unit that is more complex than a single glycoprotein molecule, as shown by the shoulders present in the radiation inactivation curves in Figures 2 and 3. Since the inactivation targets for erythrocyte and CL liposome fusion are different, the two reactions must occur by different mechanisms.

Radiation inactivation curves with shoulders such as that for Sendai–CL liposome fusion (Figures 2 and 3) may be analyzed in terms of a generalized "multiple hit–multiple target" model, in which the functional unit is assumed to consist of $m$ targets of identical size, each of which requires $n$ radiation-induced events for inactivation (Oliver & Shepstone, 1964). Many different values of $m$ and $n$ can generally be found to fit the experimental data, so that unique structural information cannot be obtained from the inactivation curves alone. If it is assumed, however, that all targets are viral glycoproteins and that a single molecule is inactivated by each hit (as suggested by the inactivation curve for Sendai–erythrocyte ghost fusion, Figure 2), then the analysis simplifies to a "multiple target–single hit" model ($n = 1$; Oliver & Shepstone, 1964).

Under this assumption, the functional unit for Sendai–CL liposome fusion at pH 7.0 consists of two to three targets of 52 ± 9 kDa (Figure 2). Under the same assumption, the functional unit calculated for Sendai–CL liposome fusion at pH 5.0, or at pH 7.0 with added gangliosides, consists of two targets of 64 ± 10 kDa (Figure 3), essentially the same value. We suggest on this basis that fusion under all three conditions occurs by a similar or identical mechanism, in contrast to the conclusions drawn by Klappe et al. (1986). These experiments do not identify whether F or HN proteins (or both) mediate the fusion, however, and in this case the involvement of HN is possible.

The only significant difference between the three Sendai–CL liposome fusion curves is the activation seen at low radiation doses at pH 7.0 in the absence of gangliosides (Figure 2). The other two conditions—addition of gangliosides or lowering of pH (Figure 3)—are both thought to increase binding of Sendai to CL liposomes (Klappe et al., 1986). Since these conditions simultaneously eliminate the activation, the increased activity may arise from a radiation-induced enhancement of the binding interaction rather than from a change in the mechanism of the fusion reaction itself.

An interpretation of a radiation inactivation curve possessing enhanced activity at low radiation doses was presented by Harmon et al. (1980) for insulin binding to insulin receptors and by Kincaid et al. (1981) for calmodulin-dependent cyclic nucleotide phosphodiesterase activity. In both cases it was assumed tht the target protein's activity was decreased or inhibited in the unirradiated state by interaction with another protein. The increase in activity at low radiation doses, i.e., the shoulder, was then attributed to the destruction of the inactivating species. The size of the inactivating species could be determined by subtraction of the experimental points from the extrapolated line. Applying this procedure to the CL liposome fusion curve in Figure 2 yields an apparent target size of 162 ± 24 kDa for the "inhibitory" functional unit. It is possible that a protein of this size, perhaps the HN spike, interferes with Sendai–CL liposome binding at pH 7.0 but not at pH 5.0 or after addition of gangliosides.

The radiation inactivation curve for hemolysis shown in Figure 2 is consistent with a single trimer or tetramer of viral glycoprotein mediating hemoglobin leakage. This assumes that a single radiation event (which inactivates a monomer, as shown in Figure 2) destroys the hemolytic activity of a much larger structure, presumably a single spike. The hemolysis curve is quite different from the curve for Sendai–CL liposome fusion (Figure 2), in which (as one interpretation, see above) 2–3 glycoprotein molecules may form one functional unit. In this case each of the three molecules is independently inactivated by separate radiation events. The three Sendai virus activities of fusion with ghosts, fusion with CL liposomes, and leakage of hemoglobin after fusion are thus mediated by different constellations of viral glycoproteins.

The target sizes for ghost and CL liposome fusion are indistinguishable for influenza virus (Figure 1), in contrast with those for Sendai virus (Figure 2). These results therefore provide no additional support for the suggestion of Stegmann et al. (1986) that these two fusion reactions occur by different mechanisms. They do not, however, rule out the possibility that a different viral glycoprotein may mediate each fusion or that the same glycoprotein unit acts in different ways on each of the two targets.

The properties of Sendai fusion with acidic liposomes (Amselem et al., 1986; Klappe et al., 1986) bear a striking resemblance to the fusion properties of vesicular stomatitis virus (VSV): the VSV reaction requires lower pH (White et al., 1983) and works especially well when phosphatidylserine is present in target liposomes (Yamada & Ohnishi, 1986). It has also been suggested that VSV binds preferentially to anionic phospholipids in the target membrane bilayer (Schlegel et al., 1985). While there seems little doubt that Sendai infection occurs most often via fusion with the plasma membrane at neutral pH (Fan & Sefton, 1978; Nagai et al., 1983), the existence of a second fusion mechanism, requiring anionic phospholipid and potentiated by low pH, might provide certain Sendai virions with a second chance at infection after they have been internalized by endocytosis but prior to their destruction in lysosomes. Thus, Sendai virus fusion with CL liposomes may not be simply an experimental artifact, as has been suggested (Klappe et al., 1986), but instead may represent an additional pathway for virus entry.

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# Cross-Linking of Streptomycin to the 16S Ribosomal RNA of *Escherichia coli*†

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ABSTRACT: [$^3$H]Dihydrostreptomycin was cross-linked to the 30S ribosomal subunit from *Escherichia coli* with the bifunctional reagent nitrogen mustard. The cross-linking primarily involved the 16S RNA. To localize the site of cross-linking of streptomycin to the 16S RNA, we hybridized RNA labeled with streptomycin to restriction fragments of the 16S RNA gene. Labeled RNA hybridized to DNA fragments corresponding to bases 892–917 and bases 1394–1415. These two segments of the ribosomal RNA must be juxtaposed in the ribosome, since there is a single binding site for streptomycin. This region has been implicated both in the decoding site and in the binding of initiation factor IF-3, indicating its functional importance.

The aminoglycoside antibiotic streptomycin binds to the 30S subunit of *Escherichia coli* at a single binding site (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973; Grisé-Miron & Brakier-Gingras, 1982). We have shown (Melançon et al., 1984) that streptomycin could be covalently linked to the 30S subunit, using the bifunctional cross-linking agent phenyldiglyoxal. Some cross-linking occurred between streptomycin and ribosomal proteins S1, S5, S11, and S13, but most of the cross-linking involved the 16S RNA.

The purpose of this study was to determine which parts of 16S RNA are involved in the interaction with streptomycin. We have cross-linked [$^3$H]dihydrostreptomycin to the 30S subunit or to the 70S ribosome of *E. coli*, and to define the sites of the 16S RNA which are cross-linked to the antibiotic, we have formed hybrids between restriction fragments of the 16S RNA gene and 16S RNA cross-linked to [$^3$H]dihydrostreptomycin, using a procedure developed by Hall et al. (1985). Single-stranded RNA and DNA were digested with a single-strand-specific nuclease, the hybrids were fractionated by gel electrophoresis, and the radioactivity in the hybrid bands was counted. Knowing the sequence of the 16S RNA gene, it is relatively simple to identify the regions of labeling of the 16S RNA from the distribution of the radioactivity among the hybrid bands. The use of various restriction enzymes, alone or in combination, allows the localization of the labeling to limited regions of 16S RNA. The method is applicable even with a very low level of labeling. An analogous procedure based on the hybridization of modified 16S RNA with DNA restriction fragments has also been used by Van Stolk and Noller (1984) to probe the 16S RNA conformation.

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