A Combined Deficiency of Vitamins E and C Causes Severe Central Nervous System Damage in Guinea Pigs\textsuperscript{1,2}

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ABSTRACT A short period of combined deficiency of vitamins E and C causes profound central nervous system (CNS) dysfunction in guinea pigs. For this report, CNS histopathology was studied to define the nature and extent of injury caused by this double deficiency. Weanling guinea pigs were fed a vitamin E–deficient or –replete diet for 14 d. Then vitamin C was withdrawn from the diet of some guinea pigs. Four diet groups were thus formed: replete, vitamin E deficient, vitamin C deficient, and both vitamin E and C deficient. From 5 to 11 d after institution of the doubly deficient diet, 9 of 12 guinea pigs developed paralysis, and 2 more were found dead. The remaining guinea pig in the doubly deficient group and all animals in the other 3 groups survived without clinical impairment until the experiment was terminated at 13–15 d. Brains and spinal cords were serially sectioned and stained for examination. Only the combined deficiency produced damage in the CNS. The damage consisted mainly of nerve cell death, axonal degeneration, vascular injury, and associated glial cell responses. The spinal cord and the ventral pons in the brainstem were most severely affected, often exhibiting asymmetric cystic lesions. Several features of the lesions suggest that the primary damage was to blood vessels. These results indicate that the paralysis and death caused by combined deficiency of vitamins E and C in guinea pigs is caused by severe damage in the brainstem and spinal cord.


KEY WORDS: \textbullet combined vitamin E and C deficiency \textbullet nutritional central nervous system injury \textbullet guinea pigs

Three essential micronutrients, vitamin E, vitamin C, and selenium, have major antioxidant functions (1). Individual deficiencies of these micronutrients have been characterized and only vitamin E deficiency that is prolonged and severe was shown to cause CNS damage (2–4).

Redundancy is a characteristic feature of the biological defense against oxidative injury. That is, there are many defense mechanisms that respond to the numerous oxidative threats, and there is overlap of these defense mechanisms so that loss of a single defense is seldom catastrophic to the organism. However, loss of more than one antioxidant mechanism can lead to a breakdown in oxidant defense with serious consequences. An example of this is the massive lipid peroxidation and liver necrosis that occurs in rats with simultaneous deficiencies of vitamin E and selenium (5,6).

To examine antioxidant micronutrient deficiency combinations that include vitamin C, animals other than rats and mice must be used because rats and mice can synthesize ascorbic acid. We carried out a series of studies using guinea pigs in which our goal was to characterize all 3 combinations of antioxidant micronutrient deficiencies. As expected, the combined deficiencies produced severe injuries. The combinations that included selenium deficiency combined with a deficiency of vitamin E or vitamin C caused damage to skeletal muscle (7,8). The combination of vitamin E and vitamin C deficiencies led to limb paralysis of sudden onset that progressed to death, usually within 12 h (9). Examination of hematoxylin and eosin–stained sections from several regions of the brain did not identify lesions that could be responsible for this fatal condition. This lack of success with random samples of the CNS indicated that examination of the entire CNS was required (9).

Our group subsequently established a collaboration with neuroscientists familiar with neuropathology and carried out an additional study. The present report describes the CNS pathology caused by simultaneous deficiencies of vitamin E and vitamin C that was found by examining serial sections through the brain and spinal cord.

MATERIALS AND METHODS

Guinea pigs. Weanling male Hartley guinea pigs (106–138 g) purchased from Elm Hill Breeding Labs were housed in plastic cages (n = 3/cage). Aspen shavings were provided for bedding. The guinea pigs were fed Torula yeast–based diets formulated and produced by Harlan-Teklad according to our specifications. The basic diet (Teklad...
CNS DAMAGE FROM DEFICIENCY OF VITAMINS E AND C

Guinea pigs were fed a vitamin E–deficient diet for 2 wk. Only 1 guinea pig fed the doubly deficient diet survived until d 13 and 15, when the study was terminated. These observations confirm that combined deficiency of vitamins E and C produces severe neurologic deficits and that comparable single deficiencies do not produce deficits (9).

**CNS lesions in guinea pigs fed the doubly deficient diet.** After fixation, the brains and spinal cords of all guinea pigs (experimental, n = 10; controls, n = 12) were removed. Brains and spinal cords of 6 doubly deficient and 2 each of the other diet groups were prepared for microscopic examination. The CNS prepared for study were chosen from guinea pigs judged to have the best perfusions and fixations. Affected guinea pigs with good perfusions and fixations were selected to represent the spectrum of clinical severity (Table 2). Sequential sections through the forebrain, midbrain, brainstem, cerebellum, and cerebrum were cut to allow egress of blood and perfusate. Then, 250–300 mL of perfusion-fixative was established through the left ventricle of the heart, the pentobarbital (65 mg/kg i.p.) before whole-body perfusion. After perfusion was established the established left ventricle of the heart, the cannula was advanced into the ascending aorta and the right atrium was cut to allow egress of blood and perfusate. Then, 250–300 mL of buffer (0.27 mol/L NaCl, 44 mmol/L dextrose, 47 mmol/L sucrose, 4.2 mmol/L CaCl₂, 3.1 mmol/L Na cacodylate, pH 7.4) was infused, followed by infusion of ~300 mL of buffered fixative (120 mmol/L sucrose, 67 mmol/L Na cacodylate, 80 g/L paraformaldehyde, pH 7.4).

The fixed head was removed and the skin was cut away before the whole head was immersed in buffered fixative for 24 h before the brain was removed. The vertebral column was isolated and immersed in buffered fixative for 24 h before the spinal cord was removed.

**Histological processing and staining.** Brains and spinal cords from 6 doubly deficient guinea pigs and 2 guinea pigs from each of the other 3 diet groups were chosen for histologic examination. Tissues from these guinea pigs were sent to NeuroScience Associates for embedding and sectioning. Serial sections at 480- or 960-μm intervals were stained by the de Omers amino cupric silver method (10) to reveal degenerating neurons and axons. Slides were counterstained with neutral red to show cell bodies. Other staining procedures used at 960-μm intervals were cresyl violet (Nissl), immunocytochemistry with antibodies to glial fibrillary acidic protein (GFAP) to visualize astrocytic perikarya and processes, and immunocytochemistry with antibodies to ionized calcium-binding adapter molecule 1 (Iba1) to visualize microglia. Staining was performed by NeuroScience Associates using their protocols (11).

**Statistics.** Statistical analyses were performed using Prism 4.0b on a G5 Macintosh computer. Values are means ± SD. Data were evaluated by 1-way ANOVA. Post hoc tests were not performed because the dietary groups did not differ.

**RESULTS**

**Clinical effects of the deficient diets.** The groups of guinea pigs had comparable weights at the start of feeding of the experimental diets (Table 1), even though some had been fed a vitamin E–deficient diet for 2 wk. Only 1 guinea pig fed the doubly deficient diet survived until d 12 of the protocol, but all fed the singly deficient and replete diets survived and gained weight.

Twelve guinea pigs were fed the doubly deficient diet; 9 had weakness or paralysis of the hind limbs between d 5 and 11 (Table 2). Two were found dead when the guinea pigs were first checked on the mornings of d 10 and 11. Only one of the doubly deficient guinea pigs remained clinically unaffected until the end of the study. It was killed on d 15.

None of the control guinea pigs, i.e., those fed a nutritionally replete diet, vitamin E–deficient diet, or vitamin C–deficient diet, developed clinical signs during the experiment. All control guinea pigs were killed between d 13 and 15, when the study was terminated. These observations confirm that combined deficiency of vitamins E and C produces severe neurologic deficits and that comparable single deficiencies do not produce deficits (9).

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>Body weights of guinea pigs fed the experimental diets¹</th>
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<tr>
<td>Diet group</td>
<td>n</td>
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<td>Nutritionally replete</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin E deficient</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin C deficient</td>
<td>4</td>
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¹ Values are means ± SD. Guinea pigs were fed nutritionally replete or vitamin E–deficient diet for 2 wk beginning at weaning. Then, on d 0 of the protocol, vitamin C was removed from the diet of some guinea pigs from each of the 2 groups, forming the 4 diet groups shown in this table.

² The weight gains of the 3 groups did not differ.
³ Only 1 guinea pig survived until d 12 in this group.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Clinical assessment of guinea pigs fed a diet deficient in both vitamins C and E¹</th>
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<td>Time consumed before impairment observed, d</td>
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</tr>
<tr>
<td>5</td>
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<td>7</td>
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</tr>
<tr>
<td>9</td>
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</tr>
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<tr>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>22³</td>
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</table>

¹ Two of the 12 doubly deficient guinea pigs were found dead on d 10 and 11 and were not studied histologically.

² Clinical severity score: 0, no impairment; 1, weakness of rear limbs only; 2, paralysis of rear limbs; 3, weakness of front limbs and paralysis of rear limbs; 4, paralysis of all 4 limbs; 5, paralysis of all 4 limbs and inability to move head.

³ Guinea pigs selected for histopathologic study of brains and spinal cords. See Table 3.
lum, and spinal cord from each guinea pig were examined. Thus, the entire CNS of each selected guinea pig was examined.

The focal pathologic changes observed in the brains and spinal cords of the doubly deficient group, although varying in size, intensity, and location from animal to animal, were similar in character. These lesions were typically seen as cystic structures (Figs. 1–4) surrounded by ring-like outlines of cells that were stained with the microglial immunostain for Iba1 (Figs. 2F and 4D). In addition, there were associated and sometimes intermixed cells stained with the astrocytic immunostain for GFAP around the cystic structures (Figs. 2E and 4B). These cystic lesions varied from <1 mm to >3 mm in diameter, and although many of these lesions were empty, some contained small aggregates of amorphous cellular debris that was unexpectedly devoid of inflammatory cells (Fig 1A).

The locations of the cystic lesions in the CNS were typically asymmetric and often unilateral. They were found predominantly in the spinal cord (Figs. 1–3) and ventral pons (Fig. 4), but some were present in hippocampus, amygdala, thalamus, hypothalamus, and medulla (Table 3). The cystic lesions in the spinal cord were distributed throughout its length (Supplemental Fig. 6).

Nissl staining revealed shrunken neurons at the edges of some cystic lesions (Fig. 1A). These shrunken neurons, along with the conspicuous absence of significant inflammation, suggest that apoptosis rather than typical necrosis may predominate in the production of these unusual lesions.

Silver staining demonstrated fragmented and degenerating neuronal processes, some comprising entire fiber bundles near cystic lesions in the pons and spinal cord (Figs. 2C, 3, and 4C). The greatest degree of axonal degeneration was observed consistently in the spinal cord in which bundles of ascending and descending fibers appeared to be affected (Figs. 2 and 3). In addition, some regions distant from the more prominent cystic structures demonstrated activated (reactive) astrocytes arranged in a ring-like fashion around small parenchymal blood vessels (Fig. 5). In addition, in some regions of otherwise well-perfused brains, the vessels retained RBC that were visualized by the silver stain (Fig. 5A). Vessels to these areas may have been occluded or constricted before perfusion, thus not allowing the RBC to be flushed out, but this issue requires further clarification. Taken together, these findings indicate that blood vessels in the CNS were affected by the double deficiency.

Cystic lesions were present in the ventral pons and/or spinal cord of all of the doubly deficient guinea pigs except one (Table 3). The guinea pig without cystic lesions was the only doubly deficient animal that did not develop clinical impairment (Table 2). None of the control guinea pigs (those fed the nutritionally replete or singly deficient diets) exhibited either the clinical signs or the pathological changes observed in the doubly deficient guinea pigs (Table 3, Supplemental Fig. 6). Thus, there appeared to be a relation between the severity and number of lesions in the CNS and the severity of clinical impairment.

**FIGURE 1** Representative Nissl-stained cross sections through lumbar segments of the spinal cords of guinea pigs fed 1 of 4 different diets: (A) deficient in vitamins E and C; (B) nutritionally replete; (C) deficient in vitamin E; or (D) deficient in vitamin C. Only the section from doubly deficient guinea pigs shows tissue damage, with large asymmetric cystic areas of cell and axon loss (marked with asterisks) that involve both the white and gray matter. A blood vessel extends to a cystic area (arrow) and islands of spared motor neurons are present within the lesion in the left ventral horn. The figure is presented in color as supplemental Figure 1.
DISCUSSION

The results presented here show that combined moderate deficiencies of vitamins E and C produced marked degenerative changes in the guinea pig CNS. None of the guinea pigs showed signs of ill health during the initial 2 wk when they were fed the vitamin E–deficient diet, but paralysis began to appear 5 d after vitamin C was removed from the diet that was already deficient in vitamin E. Thus, the affected guinea pigs were fed the vitamin E–deficient diet for 3–4 wk and the vitamin C deficient diet for 5–11 d. Consistent with the short duration of these

<table>
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<th>7</th>
<th>3</th>
<th>8</th>
<th>6</th>
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<th>22</th>
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<td>I</td>
<td>I</td>
<td>SGI</td>
<td>SI</td>
</tr>
<tr>
<td>Thalamus</td>
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<td>SG</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
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<tr>
<td>Hypothalamus</td>
<td>SNGI</td>
<td>SNGI</td>
<td>I</td>
<td>I</td>
<td>SG</td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td>S</td>
<td>SNGI</td>
<td>G</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pons</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>SNI</td>
<td>A</td>
<td>SG</td>
</tr>
<tr>
<td>Medulla</td>
<td>A</td>
<td>SNGI</td>
<td>SNGI</td>
<td>SNI</td>
<td>SNGI</td>
<td>SI</td>
</tr>
<tr>
<td>Deep cerebellar nuclei</td>
<td>S</td>
<td>SNGI</td>
<td>SNGI</td>
<td>SNI</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Spinal cord</td>
<td>S</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>S</td>
<td>I (slight)</td>
</tr>
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</table>

1 The clinical severity score was given in Table 2 and is repeated here for convenience: 0, no impairment; 1, weakness of rear limbs only; 2, paralysis of rear limbs; 3, weakness of front limbs and paralysis of rear limbs; 4, paralysis of all 4 limbs; 5, paralysis of all 4 limbs and inability to move head.

2 Key: S = silver stain, N = Nissl stain, G = GFAP stain, I = Iba1 stain, A = all stains. Underlining indicates presence of cystic lesion(s). Listing of letter for stain without underlining indicates the presence of damage without the presence of cystic lesions.

3 GFAP stain was not evaluated for guinea pig 6.

FIGURE 3 Higher magnification of a section of the silver-stained spinal cord (counterstained with cresyl violet) from a guinea pig deficient in both vitamins E and C. (A) Extensive axonal degeneration can be seen around a cystic lesion in the dorsal horn of the spinal cord (asterisk). (B) The enlargement shows fascicles of degenerating axons leaving a vacuolated region of the ventral lateral white matter (oval) and ramifying around the motor neuron cell bodies in the ventral horn of the spinal cord. The figure is presented in color as supplemental Figure 3.

FIGURE 4 Representative sections through the pons, at the level of the pontine nuclei, stained according to 4 different protocols. The left half of each pair of images is from a guinea pig deficient in both vitamins E and C, and the right half is from a nutritionally replete guinea pig. (A) The Nissl stain shows an area of tissue loss (cystic lesion indicated by an asterisk) in the ventral pons of the doubly deficient guinea pig with no apparent increase in the number of glial cell nuclei around the border of the lesion. (B) After immunoreactivity for GFAP, the lesion in the ventral pons (asterisk) is outlined in the doubly deficient guinea pig, as are the blood vessels (arrow) near the region. (C) The silver stain shows dense axonal degeneration surrounding the pontine lesion (asterisk), and one prominent bundle can be followed as degenerating axons in the pontocerebellar tract in this and adjacent sections. (D) The Iba1 stain shows microglial cells forming a border around 3 discrete lesions (asterisks) that are not present at any level in sections through the pons of a nutritionally replete guinea pig. The figure is presented at higher resolution as supplemental Figure 4.

FIGURE 5 Effects of combined vitamin E and vitamin C depletion on blood vessels in the CNS parenchyma. (A) Silver-stained spinal cord section from a doubly deficient guinea pig with a dense zone of degeneration on the right side (black patch) and widespread staining of RBC within vessels on the left side (one vessel containing them is indicated by the arrow). Such vascular retention of RBC was noted in circumscribed areas in several other guinea pigs that had cystic lesions. (B) GFAP staining of a brainstem section from a doubly deficient guinea pig shows that astroglial cells and their processes outline blood vessels. (C) At higher magnification, reactive astrocytes can be seen with enhanced GFAP immunoreactivity around blood vessels (indicated by the left end of the double-headed arrow) and along the midline of the pons compared with (D), a section through the pons from a nutritionally replete guinea pig. The right end of the double-headed arrow points to a blood vessel that does not have an accumulation of GFAP immunoreactivity. The figure is presented in color as supplemental Figure 5.
deficiencies, our previous study showed that the vitamin E and vitamin C levels in brain and spinal cord of affected guinea pigs were >50% of control levels (9). Thus, the individual deficiencies were moderate and without obvious clinical consequences, but their combined occurrence led to severe CNS lesions and death.

Characteristics of the deficiency-induced lesions. The combined vitamin E and vitamin C deficiency produced cell death with characteristics of necrosis and apoptosis. Changes occurred such as shrunken neurons, characteristic of apoptosis, as well as swollen cells with an inflammatory response, characteristic of necrosis. There were areas of apparent cell loss with and without a strong glial reaction, as assessed by the presence of increased GFAP and Iba1 in astrocytes and microglia, respectively. Lesions in which a marked gliosis was not found might have been generated by extensive apoptosis, where it would be possible that the fragmented cells overwhelmed the phagocytic response by nearby glial cells, resulting in minimal inflammation.

The minimal glial response around large brain lesions seems inconsistent with the magnitude of the insult. A potential explanation is that lesions with a minimal glial response might reflect a very early stage in the response cycle, perhaps because of the short period between the onset of the primary lesion and the occurrence of paralysis. The exact time of onset of the pathological process could not be determined in these studies, but these observations leave only a few days at best for the production of these extensive brain lesions. Experimental studies of response to brain injury showed that the first sign of a microglial response can be detected at ~12 h (12). Usually, 2–3 d are required to develop a strong microglial response (13). Significant astrogliosis response requires 3–4 d to develop (14). Considering that the clinical picture changes within 12 h (12), usually 2–3 d are required to develop a strong microglial response, it is possible that the lesions are too recent to exhibit a mature glial reaction.

Axonal degeneration was quite varied in the CNS of doubly deficient guinea pigs but was often present in fiber tracts leading to or emerging from the ventral pons, e.g., pontocerebellar fibers and pyramidal tract fibers (not shown). Degenerating axons were also seen proximal to the cystic lesions found in different regions and, occasionally, in isolated clusters in which there were no visible lesions (Fig. 3). It is possible that these isolated clusters were axonal projections of degenerating neurons associated with active focal lesions some distance away. Scattered degenerating axons were seen in some controls. They were mainly single degenerating axons, as opposed to the clusters or axonal bundles observed to be degenerating in doubly deficient guinea pigs.

Pathogenesis of the CNS lesions. Several observations in this study suggest that blood vessel injury may have been the primary cause of the CNS injury. One is the asymmetric distribution of lesions, which is characteristic of vascular injury but uncommon in pathologic metabolic processes. Another is that many vessels in doubly deficient brains were ringed with endothelial desquamation from the luminal surface, all of which is characteristic of necrosis. There were areas of apparent cell loss with and without a strong glial reaction, as assessed by the presence of increased GFAP and Iba1 in astrocytes and microglia, respectively. Lesions in which a marked gliosis was not found might have been generated by extensive apoptosis, where it would be possible that the fragmented cells overwhelmed the phagocytic response by nearby glial cells, resulting in minimal inflammation.

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The recent biochemical literature on vitamin E and vitamin C suggests a mechanism that could lead to cell injury when concentrations of both of these vitamins are low. Vitamin E scavenges free radicals in membranes; consequently, it is converted to the α-tocopheroxyl radical. In vitro studies showed that vitamin C in the water phase is able to donate a hydrogen atom to the α-tocopheroxyl radical in the membrane, regenerating α-tocopherol (15). Several enzymes can then reduce the resulting ascorbyl radical back to ascorbate (16,17). Thus, vitamins E and C were postulated to form an electron transport mechanism from NAD(P)H to potentially injurious free radicals in membranes. This would protect the membranes from free-radical injury. Breakdown of this mechanism might be responsible for injury to blood vessels in the CNS of doubly deficient guinea pigs, although this study does not identify unequivocally the cells that are the first to be injured by the double deficiency.

Vitamin E deficiency by itself was shown to lead to degeneration of the CNS and of skeletal muscle (18). After consumption of a vitamin E–deficient diet for 3 y, rhesus monkeys were observed to develop sensory loss with degeneration of axons in the posterior columns of the spinal cord, dorsal roots, and peripheral nerves (2). Only a small number of neurons showed degeneration. There was a loss of axons and myelin sheaths in posterior columns, accompanied by mild fibrillary astrocytosis. The nervous system lesions were accompanied by muscle weakness and muscle cell death. Similar results were observed in rats fed a vitamin E–deficient diet for 1 y (4).

Deficiency of vitamin C produces scurvy, which develops in guinea pigs after ~3 wk of being fed a vitamin C–deficient diet. The deficiency was shown to cause morphologic changes in vascular endothelium and smooth muscle (19). Type IV collagen and elastin decrease in scurvy, causing the connective tissue defects that are seen in blood vessels. The main effects of vitamin C deficiency are on connective tissue and endothelial cells. There are alterations in the wall of the aorta, disruption of the elastic laminae, smooth muscle cell proliferation, and focal endothelial desquamation from the luminal surface, all of which affect vascular integrity.

Thus, some of the effects that were described in vitamin C deficiency alone and in vitamin E deficiency alone are observed in the doubly deficient guinea pigs; the outstanding difference is that the double deficiency quickly produces lethal effects, whereas the single deficiencies require longer times to produce their lesser effects.

In conclusion, the studies reported here demonstrate extensive lesions in brains and spinal cords of guinea pigs fed a diet deficient in vitamins E and C. The lesions developed soon after institution of the doubly deficient diet and were present only in guinea pigs with paralysis. We speculated that the CNS damage resulted from oxidative injury to blood vessels supplying the brain and spinal cord regions involved.

ACKNOWLEDGMENT

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