First Report of the Microsporidian Infection of the Cabbageworm,
Pieris rapae crucivora BOISDUVAL
(Lepidoptera : Pieridae) in Japan

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A microsporidian was isolated from the progeny of field-collected cabbageworm larva. Its spore size was from 3.1 to 5.3 μm in length and from 1.7 to 2.3 μm in width. When spores were stimulated to extrude their polar filament by hydrogen peroxide, their length ranged from 47 to 108 μm . Schizonts underwent binary fission and multiple fission, and most of sporonts were monosporoblastic, though few were disporoblastic.

Of lepidopterous insects used, eight species including silkworm, rice stem borer and cotton leafworm were susceptible, but the site of infection and pathogenicity varied from species to species. In the case of cabbageworm, infection occurred in the midgut most severely and their death time came earliest. In cotton leafworm, infection localized in nervous tissue particularly, and in silkworm and rice stem borer though the development of disease was slow, the disease well developed in muscle, silk glands and Malpighian tubes, and advancing to other tissues gradually.

This microsporidian was identified as *Nosema mesnili* PAILLOT (1918) from its morphology, life cycle, host range and infected tissues.

INTRODUCTION

In autumn 1971, a disease prevailed within the colony of cabbageworm, *Pieris rapae*, which is a progeny of larvae collected from a field near NISSO Institute for Life Science, Oiso, Kanagawa, Japan. All insects succumbed to death before the third molting. Microsporidian infection was determined by microscopical observation. Although three microsporidians, *Nosema brassicae* (PAILLOT, 1918b), *N. mesnili* (PAILLOT, 1918a; TANADA, 1953) and *Perezia pieris* (PAILLOT, 1924b), have been recorded from cabbageworm, none of microsporidians associated with cabbageworm was not recognized in Japan.

Present paper described some pathological features of the microsporidian and discussed its taxonomical position.

MATERIALS AND METHODS

Spores collected from diseased larvae were partially purified by filtration through cheesecloth and alternation of high and low speed centrifugation. Aqueous suspension of spores was stored at 4°C in a refrigerator before use.

The inoculation experiments were accomplished against three orders of insects including fifteen species (Table 3). The insects were infected experimentally by ex-

posing them to the diet contaminated with known volume of spore suspension. Inoculum size was determined by counting spores in the suspension with THOMA's hemocytometer. Tween 20 (Wako Chemical Co., Ltd.) was added to the inoculum, when necessary, for improving the adhesiveness of suspension. Some species of insects used in this study were reared under laboratory conditions, but most species were obtained directly from field populations in the vicinity of our Institute. The inoculated stages and number of insects used are shown in Table 3. Among these insects, cabbageworm, silkworm, *Bombyx mori* LINNE, cotton leafworm, *Spodoptera litura* FABRICIUS, and rice stem borer, *Chilo suppressalis* WALKER, were rather susceptible and were studied in detail about their symptoms and progress of disease. All the inoculated insects were examined for infection after they either died or developed to adults.

The size and shape of fresh spores, which were obtained from different hosts, were compared in saline under phase-contrast microscope. Stages in the life cycle of the microsporidian were studied with smears of infected tissues stained with Giemsa. All measurements were made with ocular micrometer at a magnification of $1500\times$. The nuclei of spore, stained with Giemsa after hydrolyzing by 1N-HCl (60°C, 5 min.), were studied. The extrusion of polar filament was made by the application of 1.5% hydrogen peroxide containing 0.1 M potassium chloride, and the length of filament was measured from an enlarged picture which was taken with dark-field microscope.

To know the site of infection, tissues were dissected out of diseased larvae within 24 hours after death and observed under phase-contrast microscope. For histological observation, diseased larvae which exhibited the distinct external symptoms were fixed in Bouin, dehydrated in alcohol, embedded in paraffin wax of melting point 58°C, sectioned at $4\mu\text{m}$, and stained with Giemsa. These sections were not only made from the cabbageworm, which was the original host, but from the silkworm, cotton leafworm and rice stem borer for further studies.

Table 1. COMPARISON OF THE SIZES OF FRESH SPORES OF *N. mesnili* FROM FOUR HOST SPECIES

Host	No. of spores measured	Length (μm)		Width (μm)	
		M $\pm$ SE ^a	Range	M $\pm$ SE ^a	Range
<i>Pieris rapae</i>	50	3.90 $\pm$ 0.51 ^b	3.1–5.3	1.93 $\pm$ 0.13 ^c	1.7–2.3
<i>Spodoptera litura</i>	50	3.72 $\pm$ 0.26 ^b	3.0–4.2	2.00 $\pm$ 0.14 ^c	1.6–2.4
<i>Bombyx mori</i>	50	3.95 $\pm$ 0.32 ^b	3.2–5.1	1.83 $\pm$ 0.12 ^c	1.4–2.2
<i>Chilo suppressalis</i>	50	3.94 $\pm$ 0.29 ^b	3.2–4.5	1.89 $\pm$ 0.11 ^c	1.7–2.2

^a Mean and standard error.

^b *P. rapae* cf. *S. litura*, $P=0.20$; *P. rapae* cf. *B. mori*, $P=0.20-0.10$; *P. rapae* cf. *C. suppressalis*, $P=0.10$; *S. litura* cf. *B. mori*, $P=0.50-0.30$; *S. litura* cf. *C. suppressalis*, $P=0.20$; *B. mori* cf. *C. suppressalis*, $P=0.99$.

^c *P. rapae* cf. *S. litura*, $P=0.10-0.05$; *P. rapae* cf. *B. mori*, $P=0.05-0.02$; *P. rapae* cf. *C. suppressalis*, $P=0.20-0.10$; *S. litura* cf. *B. mori*, $P=0.001$; *S. litura* cf. *C. suppressalis*, $P=0.001$; *B. mori* cf. *C. suppressalis*, $P=0.50-0.30$.

RESULTS

Morphology and life cycle

Living spores were highly refractile and elongate ovoidal to ovocylindrical in

shape. Their sizes and shapes were rather various, and a few macrospores were also observed. As represented in Table 1, the width of spores obtained from four host species varied significantly, though the variation of the length of spores was not significant. When stained the hydrolyzed spores with Giemsa, a pair of nuclei revealing red to purple color were observed, which arranged along the major axis of spore. The extrusion of polar filament from spore was easily done by hydrogen peroxide, but by wet-dry-wet method the extrusion rate was considerably low. The length of filaments ranged from 47 to 108 μm , the average being 76 μm . This wide variation in length may be caused, in part, by incomplete extrusion of filament from spore. Pressure applied to the slip of a wet mount did not induce the extrusion of polar filament from spore, but only caused the disruption of many of them.

Fig. 1 Stages in the life cycle of *Nosema mesnili*, all from methanol-Giemsa treated smears. A, early schizont; B-G, stages in binary fission; B, binucleate schizont; C, quadrinucleate schizont; D, dividing quadrinucleate schizont; E, daughter schizont; F, chainettes; G, diplokaryon; H-J, stages in multiple fission; H, binucleate schizont; I, quadrinucleate schizont; J, octonucleate schizont; K, binucleate sporont; L, quadrinucleate sporont; M, disporoblastic stage; N, sporoblast; O, mature spores.

Stages in life cycle of this microsporidian, as were obtained from smears of midgut of the cabbageworm, are presented in Fig. 1 and their sizes are shown in Table 2. The first forms observed in infected larvae were very small schizonts (Fig. 1A). They contained the nuclei which were stained weakly by Giemsa. This microsporidian, when multiplied by binary fission, increased in size first (Fig. 1B), its paired nuclei divided, giving rise to a round quadrinucleate schizont (Fig. 1C), and then separated

into two daughter schizonts (Fig. 1E) which were smaller, round to polygonal and stained deeply. "Diplokarya" (Fig. 1G), though few in number, appeared when daughter schizonts were formed. However, since the size of diplokarya, the appearance of its nuclei and cytoplasm were so different from these daughter schizonts, it is difficult to assume that all diplokarya developed from these schizonts. Occasionally the chains of cells, which called "chainettes" (Fig. 1F), comprised from two to eight cells were found, but the chainettes were usually composed of four cells.

Whereas, this microsporidian, when underwent multiple fission, grew into much larger binucleate schizont first (Fig. 1H). Thereafter the nuclei divided to become having four or eight nuclei (Fig. 1I and J). These schizonts were different from those of binary fission not only in the way of division, but also in the size, shape, stainability, and other features of nucleus and cytoplasm. Multinucleate schizonts possibly gave rise to binucleate schizonts by division of cytoplasm. This stage seemed to pass quickly to start sporogony since these binucleate schizonts were not detected.

Considerable number of the protozoa at sporogonic stage were observed in smear slides about four days after inoculation. They were all larger and more elongate than those of schizogonic stages. Early form of sporont was a binucleate sporont (Fig. 1K) which was characterized by its elongate ovoidal form and vacuolated cytoplasm. Occasionally the nuclei divided to form a quadrinucleate sporont (Fig. 1L), which passed the disporoblastic stage (Fig. 1M) and then divided to form two sporoblasts (Fig. 1N). But it was hard to say that all the binucleate sporonts developed to the quadrinucleate form by nuclear division because of the low frequency of quadrinucleate sporont. This interpretation was supported by the fact that the sporoblast and spores did not usually arrange in pair, but distributed in smear at random. Consequently, it might be considered that most of sporoblasts are formed from monosporoblastic binucleate sporonts directly.

Table 2. THE SIZES (μm) OF *N. mesnili* AS OBSERVED IN DIFFERENT TYPE OF FISSION^a

Stages	Binary fission type			Multiple fission type		
	No. of Sample	$M \pm SE^b$		No. of Sample	$M \pm SE^b$	
		Length	Width		Length	Width
Early schizont	7	1.62 ± 0.32	1.34 ± 0.18		not detected	
Binucleate schizont	29	3.93 ± 0.64	3.69 ± 0.62	7	6.03 ± 0.94	5.52 ± 0.81
Quadrinucleate schizont	20	4.48 ± 0.64	4.22 ± 0.51	11	8.34 ± 1.41	7.05 ± 1.07
Octonucleate schizont		not detected		6	9.49 ± 0.64	8.51 ± 0.78
Diplokaryon	8	3.68 ± 0.27	3.25 ± 0.41		not detected	
Binucleate sporont	33	6.17 ± 0.90	2.40 ± 0.81	2	5.84 ± 0.14	2.33 ± 0.08
Quadrinucleate sporont	14	7.51 ± 1.17	2.44 ± 0.41		not detected	
Disporoblastic stage	13	8.62 ± 1.40	2.14 ± 0.31		not detected	
Sporoblast	14	3.77 ± 0.54	2.16 ± 0.33	7	3.87 ± 0.15	2.00 ± 0.14
Mature spore	47	4.00 ± 0.36	2.30 ± 0.39	25	3.85 ± 0.55	1.99 ± 0.13

^a The material was obtained from cabbageworm.

^b Mean and standard error.

Host range and pathogenicity

Although cockroach and sawfly did not receive the infection of this morcosporidian, it invaded insects of several species from six lepidopterous families below, Pieridae, Noc-

tuidae, Heliodinidae, Pyralidae, Bombycidae and Arctiidae. Consequently, it was considered that this microsporidian had rather wide host spectrum. But as shown in the response of insects of Noctuidae, the susceptibility to the microsporidian was extremely varied even among the insects which are belonged to the same family.

The pathogenicity of the parasite was investigated against cabbageworm and silkworm in relation to host age. In cabbageworm, there was no clear difference of

Table 3. THE PATHOGENICITY AND CROSS INFECTIVITY OF *N. mesnili*

Host insect	Larval instar at the time of inoculation	Dose (spores/larva)	No. of insect used	Percent diseased	Days until death	
					Range	50% died
LEPIDOPTERA						
Pieridae						
<i>Pieris rapae</i>	1st	8.3×10^6	15	60	5—10	7
		8.3×10^3	15	73	7—14	8
		8.3×10	15	0		
	2nd	3.0×10^7	10	80	5—9	6
	3rd	1.9×10^6	48	44	4—10	7
	4th	1.8×10^6	50	52	5—13	9
	5th	2.0×10^6	45	24	3—13	10
Bombycidae						
<i>Bombyx mori</i>	1st	1.4×10^6	20	95	5—20	9
	2nd	5.2×10^6	20	95	6—42	19
	3rd	1.0×10^6	5	100	20—41	38
		1.0×10^2	8	25	51—70	51
	5th	1.6×10^6	5	60	6—12	10
Noctuidae						
<i>Spodoptera litura</i>	2nd	8.0×10^6	20	95	3—22	5
<i>Plusia agnata</i>	3rd—5th	3.0×10^7	5	20	5	5
<i>Plusia nigrisigna</i>	3rd—4th	3.0×10^7	5	20	17	17
<i>Leucania separata</i>	2nd—3rd	3.0×10^7	10	0		
<i>Mamestra brassicae</i>	2nd	3.0×10^6	10	0		
Pyralidae						
<i>Chilo suppressalis</i>	2nd	3.0×10^7	40	60	10	30
Heliodinidae						
<i>Plutella maculipennis</i>	2nd—3rd	1.0×10^6	15	47	4—28	9
Arctiidae						
<i>Spilarctia inaequalis</i> i.	4th—5th	1.0×10^6	15	8	12	12
Tineidae						
<i>Nemapogon granella</i>	middle to late	3.0×10^{7a}	20	0		
HYMENOPTERA						
Tenthredinidae						
<i>Allantus luctifer</i>	2nd	8.0×10^6	10	0		
BLATTARIA						
Blattidae						
<i>Periplanata americana</i>	1st	3.0×10^{7a}	10	0		
<i>Periplanata fumiferana</i>	1st	3.0×10^{7a}	10	0		
Blattellidae						
<i>Blattella germanica</i>	1st	3.0×10^{7a}	10	0		

^a Spores/g. diet.

susceptibility to the microsporidian among ages. They died six to ten days after inoculation, irrespective of their age at inoculation. Cabbageworm died fairly fast by the microsporidian, but the rate of infection was not high. Considerable number of insects succumbed to death by invasion of bacteria prior to the occurrence of microsporidiosis.

Comparing to cabbageworm, the development of disease in the silkworm larvae was rather slow, but the infection rate was high. The silkworm, when inoculated with the microsporidian after the third instar, many larva were able to develop to pupa or moth. However, they were found to be infected systemically.

The host insects parasitized by this microsporidian might be classified into three groups by the length of period from inoculation to death. The first group which included cabbageworm, original host, died earliest, usually about one week. The second group comprised cotton leafworm and diamond back moth, *Plutella maculipennis* CURTIS. They took one to two weeks until death. The last group, to which silkworm and rice stem borer were belonged, died three weeks to more than one month after inoculation. The site of infection also varied among insects of these groups, which will be described in detail later.

Symptoms

The external and internal symptoms of the disease which were found in four kinds of insects, are summarized as below. As is the case of microsporidian disease of other insects, infected larva first ceased to feed and immobilized, their growth and development were delayed markedly and then stopped. Diarrhea was recognized in some of them. At the late stage of infection, insects could not feed, and the change of coloration also occurred. When they were close to death, the body withered heavily and the depletion of internal moisture was observed. Body of dead larvae became soft but their integument itself was still tough.

The larva did not show distinct internal symptoms until the disease developed to late stage. At the late stage, heavily infected tissues were so debilitative and its luster disappeared. The tissues, particularly Malpighian tubes and silk glands which permitted severe infection, became whitish by the accumulation of spores. Moreover, as a result of cessation of feeding, the depletion of internal moisture and the reduction of hemolymph were also observed. Other remarkable signs of disease were observed in two insect species. In silkworm, many of larvae, which were inoculated at the last larval instar, died at the stage of mounting and they suffered the complication of bacterial infection. On the other hand, the larvae of cotton leafworm showed slight paralysis which was resulted from the ingestion of high dose of the spores.

Infected tissues

As represented in Table 4, the site of infection varied from host to host and closely related to the virulence of the microsporidian. In cabbageworm which died within the shortest period, the main sites of infection were midgut, muscles, cardiac tissues, Malpighian tubes and silk glands, and the parasite developed in midgut epithelium fastest and most severely.

In cotton leafworm which died somewhat slowly so that it was classified into the second group, the nervous tissues were parasitized particularly, though some spores were observed in muscles and hemolymph, and they died before the time that the de-

velopment of parasites in the other tissues severely occurred.

In both silkworm and rice stem borer in which the disease was chronic, the parasitization was systemic. In the early stage of disease, the infection occurred in mu-

Table 4. COMPARISON OF TISSUES INFECTED WITH *N. mesnili*^a

Tissue	Host insect			
	<i>P. rapae</i>	<i>S. litura</i>	<i>B. mori</i>	<i>C. suppressalis</i>
Hemolymph	+	+	+	
Blood cell	+	—	—	
Muscle	+++	+	+++	+++
Nervous tissue	+	+++	+	
Foregut	+	—	+	+
Midgut	+++	—	+	+
Hindgut	+	—	+	+
Salivary gland	+		+	+
Silk gland	+++	—	+++	+
Malpighian tube	+++	—	+++	+
Adipose tissue	+	—	+	+
Cardiac tissue	+++	—	+++	+
Trachea	+	—	+	+
Oenocyte	+		+	+
Epidermal cell	+		+	+
Testis	+	—	+	

^a Number of + signs represents relative degree of infection; — sign indicates no infection.

Fig. 2 Sections and fresh preparation of larva infected with *N. mesnili*. A, Section of heavily parasitized midgut and Malpighian tube of cabbageworm ($\times 100$); B, Fresh preparation of nerve fiber of cotton leafworm filled with the spores of *N. mesnili* ($\times 400$); C, Section of midgut and infected muscles of silkworm ($\times 100$); D, Section of lightly infected midgut and infected muscles of rice stem borer ($\times 100$). MG, midgut; MT, Malpighian tube; NF, nerve fiber; Mus, muscle; Nm, *Nosema mesnili* spore. Nm spores were showed as dark area.

scles mainly. At this stage, though silk glands and Malpighian tubes were also parasitized, the parasite hardly attacked midgut epithelium. The site of infection in this type of host extended as the larvae survived longer, and finally the infection spreaded into foregut, hindgut, nervous tissues, genitals, trachea, epidermal cells, adipose tissues and oenocytes.

DISCUSSION

Recently, SPRAGUE and VERNICK (1971) reported about the characters of three disporous genera, *Nosema*, *Glugea* and *Encephalitozoon*. By this classification, sporont of *Nosema* sp. divides to produce two sporoblasts, and the nuclei pair in diplokaryon arrangement throughout most of the life cycle, including the spores. And both sporonts of *Glugea* and *Encephalitozoon* also divide to produce two sporoblasts, and the nuclei of both species do not pair during most of the life cycle. And in the case of *Glugea* infection, the parasite produces a cell hypertrophy tumor or xenoma.

The sporont of microsporidian studied here seemed to develop into spores through two possible ways as below, (1) majority of sporonts developed to spores directly without division and (2) a few sporonts divided into two sporoblasts which developed into spores. And this parasite did not produce cell hypertrophy and the nuclei showed the diplokaryon arrangement in most stages in electron microscopical study (ISHIHARA, unpublished). Consequently, this microsporidian should be classified into genus *Nosema*.

For identification of this microsporidian, it must be compared with species of genus *Nosema* which recovered from the cabbageworm previously. Three species of microsporidians are reported, that is, *Nosema brassicae* (*Perezia legeri*) by PAILLOT (1918b), *Nosema mesnili* (*Perezia mesnili*) by PAILLOT (1918a) and TANADA (1953), and *Perezia pieris* by PAILLOT (1924b). These are classified according to the infected tissues, the existence of vacuole in spore and the development of schizont as follows.

Nosema brassicae: The spore size is 4–5 μm in length and 1.2–2 μm in width. The spore has a distinct vacuole near the base of the polar filament. It usually parasitizes the oenocytes, adipose tissues and certain giant cells of the blood, but occasionally gives rise to systemic infection. PAILLOT (1924a, 1933) was unable to transmit this protozoa by feeding the spore to the larvae. This microsporidian has schizonts which form "chainettes" of two to four cells, and some schizonts have several nuclei, 4 to 11 in number, and are generally large and variable in shape.

Nosema mesnili: The spore size is 3.5–4.4 μm in length and 1.5–2.5 μm in width. The length of polar filament is 55 μm on an average. This microsporidian parasitizes mainly Malpighian tubes and silk glands, but other tissues such as adipose tissues, muscles and intestinal epithelium are also parasitized. The spore is binucleate and has no vacuole. Both microspores and macrospores are found. This species forms "chainettes" of four cells but not more than four, and also produces schizonts with 4, 6 and 8 nuclei.

Perezia pieris: The spore size is about 4.5 μm in length and about 2 μm in width. The spore is initially mononucleate and becomes binucleate when it matures. It mainly parasitizes Malpighian tubes and silk glands, but adipose tissues, muscles and intestinal epithelium are also parasitized. Multinucleate schizonts are rarely found in this species and the number of nuclei is never observed to exceed four.

On the other hand the microsporidian isolated from cabbageworm by the author, had spore which size was 3.1–5.3 μm in length and 1.7–2.3 μm in width, parasitized

midgut epithelium, Malpighian tubes, muscles and silk glands of cabbageworm. The spore had no vacuole and both microspores and macrospores were found. This microsporidian formed "chainettes", and also produced multinucleate schizont. Transmission to the larvae of this species was performed easily by oral inoculation. According to these characteristics described above, this microsporidian resembles *Nosema mesnili* most.

From the study of spore size of *Perezia fumiferanae* and *Nosema cerasivoranae*, THOMSON (1960) concluded that there was no significant variation in mean size of a single species of microsporidian developing in different host species. Such a result was also obtained by UNDEEN and MADDOX (1973) as to the spore size of *Nosema algerae*, more recently. On the contrary, the reports as to the change of spore size in alternate host were accumulated gradually (TANAKA et al., 1971; BROOKS and CRANFORD, 1971). In this microsporidian, however, there was no significant variation in the dimension of the spore though the width of spores varied slightly when the microsporidian developed in different host species.

There are many interesting problems in the relationship between the pathogenicity and the infected tissues. Of the susceptible insects, cabbageworm succumbed to death early after inoculation and parasitized at the midgut epithelium most severely. The debility of this site caused diarrhea and often permitted the secondary invasion of intestinal bacteria which brought the insect to death quickly. Cotton leafworm, however, did not come to death so soon. But the author observed the insects when fed high dose of spores, revealed slight paralysis-like symptom. The microscopical examination of infected tissue showed that spores were found in nervous tissues first, that was four days after inoculation. As cabbageworm did not show paralysis even though the microsporidian heavily infected midgut cells, the affinity to nervous tissue of this microsporidian may be the reasons of paralysis-like action. Though the infection also occurred in the muscles, the death of cotton leafworm might be caused by gradual debility of the whole tissues resulted from the paralysis and the cessation of feeding. Therefore they survived longer than cabbageworm after challenge of the microsporidian. The third type insects to which silkworm and rice stem borer are belonged died very slowly and the development of disease was chronic. Though they were eventually infected with the microsporidian systemically, the organs such as midgut and nervous tissue were still exempted from the attack of parasites. These insects continued feeding till the late stage of infection, and the disease became chronic. Generally, the insects which died later, were infected at more kinds of tissues than insects died earlier.

Although cross infection test is an important mean for classifying microsporidians, there are relatively few reports that investigated the cross infectivity or host range of microsporidians concerned. It is necessary to study further and to accumulate data of cross infection for taxonomic criterion. And in that case, it is preferable to establish a standard method of inoculation, and to select the species which is extensively distributing over the world as the assay insect. The microsporidian of cabbageworm had rather wide host spectrum which includes eight species of lepidopterous insects, but even in the same family of insects the susceptibility to the microsporidian was varied remarkably from species. In Noctuidae, while cotton leafworm and cabbage looper, *Plusia* sp., were susceptible, cabbage armyworm, *Mamestra brassicae* LINNE, and armyworm, *Leucania separata* WALKER, were quite immune. To complete the infection to the host larva, the following processes must be overcome, (1) the spore invades into the

digestive tract of host and germinates under proper conditions of gut, say pH, kind of ions and so on, (2) emerged sporoplasm invades the midgut cell, (3) the sporoplasm escapes from disintegration by enzymes contained in gut juice, and (4) the parasite has an affinity to host cells. The study of the mechanism of host specificity must be further investigated from the view said above.

Concerning the transovum and transovarial transmission of this microsporidian, preliminary investigation was done. The author was able to transmit this disease to the progenies from the infected parents of diamond back moth, and the schizont-like stages were found in the sections of the eggs deposited by the infected moth of rice stem borer. Consequently, though further studies must be conducted, it might be expected that the transovarial or transovum transmission of this microsporidian occurs in various species of insects.

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