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SYNERGISTIC ACTION OF CHLORPYRIFOS-METHYL AND JUVENILE HORMONE ANALOGUE AGAINST LARVAL CARBOHYDRATE AND PHOSPHATASE BIOCHEMISTRY OF *EPHESTIA CAUTELLA* WALKER (LEPIDOPTERA: PYRALIDAE)

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ABSTRACT: Sub-lethal concentrations (i.e.1,2 and 4ppm) of a synergistic mixture chlorpyrifos-methyl + methoprene in the ratio of 9:1 when exposed to third instar larvae of almond moth, *Ephestia cautella* Walker caused a significantly dose-dependent ($p < 0.05$) reduction in glycogen level and alkaline phosphatase activity and a significantly dose-dependent ($p < 0.05$) enhancement in the reducing sugar lavel and the activity of acid phosphatase. The highest concentration i.e. 4ppm of this mixture exerted severe effect on glycogen and reducing sugars level as well as on the activities of acid and alkaline phosphatases, which along with other perturbed biochemical parameters by this synergist leads to death of the larva.

KEYWORDS: *Ephestia cautella*, Chlorpyrifos-methyl, Methoprene, Haemolymph, Fat body

INTRODUCTION

The control of insect pests is a puzzling problem since many decades. The almond moth, *Ephestia cautella* (Walker) is a serious pest of stored cereals, nuts, dried fruits, stored vegetables and cereal products in India, Florida, Northern Europe, South America, South California, Turkey, United States and other tropical and temperate regions of the world.[12,53,45] Its larval stages cause serious damage to wheat, wheat-flour, maize, cocoa beans, citrus pulp, bulk grain, fig, hazelnuts, ground nuts, nuts, dried fruits, pulses, peanuts, etc.[53,45,3] Considerable damage is caused by the larval feeding and by contaminating stored food with dead bodies and their own products e.g. excreta, exuviae, webbing, frass, silk and feces.[1,26]

Numerous Investigations have shown that synthetic organic insecticides (organochlorines, organophosphates, natural plant products and synthetic pyrethroids) affect the biochemical constituents of various tissues in insects. Most of them impose adverse effects on non-target organisms including parasites and predators, development of resistance and even contaminate the whole environment. Hence, as a safer substitute, organic insecticides like chlorpyrifos-methyl synergized with insect growth regulators (IGRs) is the need of the present day for the effective control of lepidopterous pests in general and almond moth, *Ephestia cautella* in particular.

Chlorpyrifos-methyl (O, O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate)

is an organophosphorous insecticide effective against a wide range of insect pests in crops of commercial importance. Its limited environmental persistence and lack of cross resistance makes chlorpyrifos-methyl a more attractive prospect than DDT for indoor residual spraying.[57] In addition, chlorpyrifos-methyl has been found to be much effective against *P. interpunctella*[7] and *Rhyzopertha dominica.*[51]

Insect growth regulators (IGRs), also called "Third-Generation Pesticides", mimic insect's hormone and regulate the insect population through the disruption of moulting and metamorphosis.[65,44] IGRs have been shown to generally have a good margin of safety for most non-target biota, as they display a very low toxicity for human and other mammals, are readily biodegradable (i.e. very low persistence in the environment), highly toxic to target insects, and leave no hazardous residues, making JHAs very useful in food preservation and storage.[61]

Methoprene a Juvenile Hormone Analogue (JHA) was first introduced into the market as Altosid™ (EPA Reg. No. 2724-393) in 1975[61], then later re-registered as Diacon™ (EPA Reg. No. 2724-788) in the 1980.[9,37] It is also registered in Australia for use in cereal grains, excluding malting barley, to control strains of the grain borer, *R. domonica*, which are resistant to synthetic pyrethroids.[20] It has been useful in combinations with older neurotoxic pesticides particularly for control of pest species that have developed resistance to such pesticides. Methoprene is a long chain hydrocarbon ester considered to have higher potency and better field stability than do naturally occuring juvenile hormone.[35] It is a selective, stable and potent larvicide; an ether and diunsaturated fatty acid ester; and its toxicity to insects is manifest through interference with metamorphosis, a process without parallel in mammals. It is non-persistent and non-toxic to mammals and presents no long-term hazards to other species at recommended application rates.

Methoprene is effective on stored product insects, including *T. castaneum*[4,40]; *T. confuse*[39], *O. surinamensis*[40,10], *R. dominica*[44,10], *C. cephalonica*[58,59,60] as well as many others.

No doubt, the present study of the almond moth, *E. cautella* treated with synergistic mixture of chlorpyrifos-methyl and methoprene may reduce the problem of pest resistance and kill or disrupt the physiological processes of the target pest by perturbing the metabolic flux of the larva. This will explore information whether the use of less amount of chlorpyrifos- methyl in synergistic mixture is able to elicit same toxic response when used singly.

MATERIALS AND METHODS

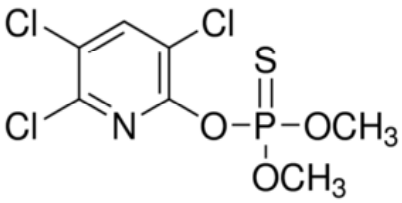
The almond moth, *Ephestia cautella*(Walker) (Lepidoptera: Pyralidae) was collected from the go-downs of Central Warehouse Corporation, Nandanagar, Gorakhpur, U.P.; Food Corporation of India, Sardarnagar, Gorakhpur, U.P.; State Warehouse Corporation, Chauri Chaura,

Gorakhpur, U.P. and State Warehouse Corporation, Sahjanwa, Gorakhpur, U.P.

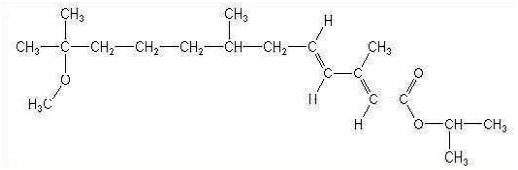
A rich standard culture of this insect was maintained in the laboratory on a normal dietary medium composed of coarsely ground wheat (*Triticum aestivum*) mixed with 5% (w/w) yeast powder and10% (w/w) glucose inside large glass containers (150 mm diameter, 200 mm height) at a temperature of 26 ± 1^0C, relative humidity 93 ± 5% and a light regime of 12 hr light and 12 hr darkness.

From the above culture whenever needed, newly emerged males and females were transferred to oviposition glass chambers (35 mm diameter, 200 mm height). Since, *E. cautella* individuals do not feed during their adult stage, no food was provided to them during their confinement in these vessels. Eggs laid by the females were collected and then placed in glass chambers (consisting of 250 ml beakers) for hatching.

A mixture of two insecticides i.e. chlorpyrifos-methyl (an organophosphate compound) and methoprene (a juvenile hormone analogue), in the ratio of 9:1 were utilized throughout the investigation. Chlorpyrifos-methyl (0,0-dimethyl 0-(3,5,6-trichloro-2-pyridyl), Molecular formula:$C_7H_7Cl_3NO_3PS$, 98.5% (a.i.) and methoprene (isopropyl (2E, 4E)-11-methoxy-3, 7, 11- trimethyl-2-1,4-dodecadienoate) molecular formula: $C_{19}H_{34}O_3$ (7.4% cis and 90.4% trans) 97.8% (a.i.) used throughout the investigation were obtained from AccuStandard, Inc.125 Market Street, New Haven, CT 06513 and have the following structural formula:



Chlorpyrifos-methyl



Methoprene

Toxicity experiments of chlorpyrifos-methyl, methoprene and their mixture in the ratio of 9:1 against ontogeny of *Ephestia cautella*, at various dose levels have been reported.[13,14,16,18] For biochemical estimations, out of various concentrations of the above mentioned insecticides only such concentrations i.e. three from the mixture of chlorpyrifos-methyl + methoprene in ratio of 9:1 (1, 2 and 4 ppm) were selected, which allowed the larvae to survive and develop but caused considerable effect in the internal biochemistry of the larva that could be easily detected and assessed to prove the effectiveness of this mixture chlorpyrifos-methyl + methoprene in the ratio of 9:1 as chemical control measures against this lepidopterous pest.

So, freshly hatched larvae were allowed to feed on a normal dietary medium

(kept inside 250 ml beakers) for 13 days. On the 14th day, 25 third instar larvae were transferred to each similar rearing chambers containing dietary medium mixed with 1, 2 and 4 ppm concentrations of the mixture of chlorpyrifos-methyl + methoprene in ratio of 9:1 and were allowed to feed for 10 days. 25 larvae were also kept as control with each set of experiment.

On the completion of 23 days, 10-15 larvae from each set, experimental as well as control were taken out. From these groups of larvae, haemolymph and fat body tissues were separately collected.[18,14] The entire programme includes biochemical estimation of glycogen, reducing sugar levels and activity of acid and alkaline phosphatases in haemolymph and fat body tissues of the larva of almond moth, *Ephestia cautella* treated with sublethal concentrations of the mixture of chlorpyrifos-methyl + methoprene in the ratio of 9:1 as well as control.

Glycogen and reducing sugar were estimated according to the method of Van der Vies[63] and Folin and Wu[32] respectively. Anthrone reagent was used for glycogen estimation while for glucose estimation, alkaline copper reagent and phosphomolybdic acid reagent were used. Acid and alkaline phosphatase activity in haemolymph and fat body was determined according to the method of Andersch and Szcypinski[6] as modified by Bergmeyer (1967) using p-nitrophenyl phosphate as substrate.

Results have been expressed as the mean ± s.e. of six replicates. Significant differences between treatment groups, in order to show dose dependence, were determined by one way analysis of variance (one way ANOVA).[50]

RESULTS AND DISCUSSION

Sublethal concentrations (1, 2 and 4 ppm) of the mixture of chlorpyrifos-methyl + methoprene (9:1) caused a significantly dose-dependent reduction in the levels of glycogen (Table 1) and a significantly dose-dependent enhancement in the levels reducing sugar (Table 2) in both the tissues of the larva.

In case of untreated larvae, the glycogen level was 2.225 and 14.720 mg/g in haemolymph and fat body respectively. The maximum decrease in glycogen level in haemolymph (20% of the control value) and fat body (27% of the control value) was recorded in larvae treated with 4 ppm concentration ofthe mixture of chlorpyrifos-methyl + methoprene (9:1). Glycogen levels, in haemolymph, were reduced to 71 (1.575 mg/g), 44 (0.981 mg/g) and 20% (0.453 mg/g) of the control value, while these levels, in fat body, were reduced to 72 (10.541 mg/g), 47 (6.922 mg/g) and 27% (3.987 mg/g) of the control value following treatment with 1, 2 and 4 ppm concentrations of this mixture respectively (Table 1).

The level of reducing sugar, in control larvae, was 2.718 and 1.059 mg/g in haemolymph and fat body respectively. The concentration of 4 ppm of the mixture of chlorpyrifos-methyl + methoprene (9:1)

Table-1. Changes in the levels of glycogen in the haemolymph and fat body of the larva of *E. cautella* treated with the mixture of chlorpyrifos-methyl + methoprene (9:1)

Concentration of chlorpyrifos-methyl + methoprene (9:1) (ppm)	Glycogen# (mg/g, wet wt.)	
	Haemolymph	Fat body
Control (untreated)	2.225 ± 0.101 (100)	14.720 ± 0.433 (100)
1	1.575 ± 0.067 (71)	10.541 ± 0.291 (72)
2	0.981 ± 0.046 (44)	6.922 ± 0.159 (47)
4	0.453 ± 0.016 (20)	3.987 ± 0.084 (27)

#Values are expressed as the mean ± s.e. of six replicates.
Values in the parentheses indicate the percentage change with control values taken as 100%.
Analysis of variance showed that the response to the mixture of chlorpyrifos-methyl + methoprene (9:1) was dose-dependent $p < 0.05$.

caused a maximum enhancement in the amount of reducing sugar which was 217% in haemolymph and 240% in fat body with respect to their control values. The amounts of reducing sugar, in haemolymph, were increased to 140 (3.812 mg/g), 183 (4.987 mg/g) and 217% (5.910 mg/g) of the control value while these levels, in fat body, were enhanced to 135 (1.434 mg/g), 173 (1.831 mg/g) and 240% (2.543 mg/g) of the control value following treatment with 1, 2 and 4 ppm concentrations of this mixture respectively (Table 2).

Table-2. Changes in the levels of reducing sugar in the haemolymph and fat body of the larva of *E. cautella* treated with the mixture of chlorpyrifos-methyl + methoprene (9:1)

Concentration of chlorpyrifos-methyl + methoprene (9:1) (ppm)	Reducing sugar#(mg/g, wet wt.)	
	Haemolymph	Fat body
Control (untreated)	2.718 ± 0.070 (100)	1.059 ± 0.086 (100)
1	3.812 ± 0.127 (140)	1.434 ± 0.020 (135)
2	4.987 ± 0.209 (183)	1.831 ± 0.359 (173)
4	5.910 ± 0.237 (217)	2.543 ± 0.049 (240)

#Values are expressed as the mean ± s.e. of six replicates.
Values in the parentheses indicate the percentage change with control values taken as 100%.
Analysis of variance showed that the response to the mixture of chlorpyrifos-methyl + methoprene (9:1) was dose-dependent $p < 0.05$.

Changes in acid phosphatase activities in haemolymph and fat body tissues of the larva of *E. cautella* treated with sublethal concentrations (1, 2 and 4 ppm) of the mixture of chlorpyrifos-methyl + methoprene (9:1) ratio have been represented in (Table 3). This mixture caused a significantly dose-dependent increase in the activity of acid phosphatase in both the tissues of the larva. In the control larvae, the acid phosphatase activity was 0.572 and 2.490 µ moles substrate hydrolyzed /30 min /mg protein in haemolymph and fat body respectively. The maximum enhancement in acid phosphatase activity in haemolymph (361% of the control value) and fat body (336% of the control value) was observed in larvae treated with 4 ppm concentration of the mixture of chlorpyrifos-methyl + methoprene (9:1) ratio. Acid phosphatase activity, in haemolymph, was increased to 169 (0.969 µ mol), 261 (1.495 µ mol) and 361% (2.067µ mol) of the control value while the activity of this enzyme, in fat body, was enhanced to148 (3.708µ mol), 241 (6.005µ mol) and 336% (8.371µ mol) of the control value following treatment with 1, 2 and 4 ppm concentrations of this mixture respectively (Table 3).

Table-3. Changes in acid phosphatase activity in haemolymph and fat body of the larva of *E. cautella* treated with themixture of chlorpyrifos-methyl + methoprene (9:1)

Concentration of chlorpyrifos-methyl + methoprene (9:1) (ppm)	Acid phosphatase#	
	Haemolymph	Fat body
Control (untreated)	0.572 ± 0.027 (100)	2.490 ± 0.038 (100)
1	0.969 ± 0.049 (169)	3.708 ± 0.036 (148)
2	1.495 ± 0.047 (261)	6.005 ± 0.085 (241)
4	2.067 ± 0.058 (361)	8.371 ± 0.078 (336)

#The activities are given as µ moles of substrate hydrolyzed/ 30 min/ mg of protein and expressed as mean ± s.e. of six replicates.

Values in the parentheses are the percentage change with control values taken as100%.

Analysis of variance showed that the response to the mixture of chlorpyrifos-methyl + methoprene (9:1) was dose-dependent $p < 0.05$

Sub-lethal concentrations i.e. 1, 2 and 4 ppm of the mixture of chlorpyrifos-methyl + methoprene (9:1) caused a significantly dose-dependent reduction in the activity of alkaline phosphatase in both the tissues of the larva. In the control larvae, the alkaline phosphatase activity was 0.447 and 2.427 µ moles substrate hydrolyzed /30 min /mg protein in haemolymph and fat body respectively. The maximum decrease in alkaline phosphatase activitiy, in haemolymph (29% of the control value) and

fat body (40% of the control value) was recorded in larvae treated with 4 ppm concentration of the mixture of chlorpyrifos-methyl + methoprene (9:1) ratio. Alkaline phosphatase activity, in haemolymph, was reduced to 70 (0.314µ mol), 50 (0.227µ mol) and 29% (0.131µ mol) of the control value while its activity, in fat body, was reduced to 84 (2.039µ mol), 66 (1.593µ mol) and 40% (0.973µ mol) of the control value following treatment with 1, 2 and 4 ppm concentrations of this mixture respectively (Table 4).

Table-4. Changes in alkaline phosphatase activity in haemolymph and fat body of the larva of *E. cautella* treated with the mixture of chlorpyrifos-methyl + methoprene (9:1)

Concentration of chlorpyrifos-methyl + methoprene (9:1) (ppm)	Alkaline phosphatase#	
	Haemolymph	Fat body
Control (untreated)	0.447 ± 0.012 (100)	2.427 ± 0.312 (100)
1	0.314 ± 0.007 (70)	2.039 ± 0.044 (84)
2	0.227 ± 0.008 (50)	1.593 ± 0.028 (66)
4	0.131 ± 0.005 (29)	0.973 ± 0.031 (40)

#The activities are given as µ moles of substrate hydrolyzed/ 30 min/ mg of protein and expressed as mean ± s.e. of six replicates.

Values in the parentheses are the percentage change with control values taken as 100%.

Analysis of variance showed that the response to the mixture of chlorpyrifos-methyl + methoprene (9:1) was dose-dependent $p < 0.05$.

Synergists are among the most straightforward tools for overcoming metabolic resistance because they can directly inhibit the resistance mechanism itself [46]. A combination of chlorpyrifos-methyl and methoprene in the ratio of 6:1 ppm caused significant effect in case of the flat grain beetle, *C. pusillus* (Schönherr), *S. zeamais*, *T. castaneum* and *P. interpunctella*[8]. In Australia also methoprene was combined with organophosphates that caused effective control of *S. oryzae* in wheat[48]. Similarly, chlorpyrifos-methyl plus methoprene (10 + 1) ppm was found to be effective against the major grain beetles[21] i.e. *S. zeamais*, *S. oryzae*, *R. dominica*, *T. castaneum* and *O. surinamensis* while chlorpyrifos-methyl at 10 ppm plus s-methoprene 0.6 ppm controlled all the strains i.e. *S. oryzae*, *T. castaneum*, *O. surinamensis* and *C. ferrugineus* except for methoprene-resistant *R. dominica*[23].

In the present study, it was observed that the main advantage of chlorpyrifos-methyl plus methoprene, when mixed in the ratio of 9:1, is that methoprene enhances the toxicity of chlorpyrifos-methyl i.e. it behaves in a synergistic way. The probable

cause of this synergism may be considered to be the combination of different mode of action, physical (desiccation) and chemical (toxicity) as reported[8] in case of *T. castaneum, C. pusillus, S. zeamais* and *P. interpunctella* following exposure of a mixture of chlorpyrifos-methyl and methoprene in the ratio of 6: 1 and *S. zeamais, S. oryzae, R. dominica, T. castaneum* and *O. surinamensis* following treatment[21] of a mixture of these two compounds in the ratio of 10 : 1.

Carbohydrates are one of the most essential biochemical constituents of insect tissues, many of which support optimum growth, development, reproductive activity and survival of individual species[19,38,66,33]. Chlorpyrifos-methyl caused a significantly dose-dependent ($p < 0.05$) decrease in glycogen level and a significantly dose-dependent ($p < 0.05$) enhancement in reducing sugar level in hemolymph as well as fat body tissues of the larva of this pest[18]. A decrease in glycogen reserve has also been reported in haemolymph and fat body tissues of *Bombyx mori* larva exposed to fenitrothion and ethion[4] , in the adults of *Tribolium castaneum* treated with malathion[47] and in the larvae of *Tribolium castaneum* following exposure of malathion[49]. Both lethal and sublethal concentrations of fenitrothion and ethion registered significant depletion in fat body glycogen reserves followed by concomitant increase in fat body phosphorylase and trehalase activities[43]. Increase in glycogen phosphorylase activity and decrease in glycogen content indicated increased glycogenolysis at tissue level. A significant decrease in glycogen reserves with a significant enhancement in reducing sugar content, in this investigation, may be ascribed to the decreased activity of glycogen synthetase and/or increased glycogenolysis, perhaps resulting from the enhanced activity of glycogen phosporylase to encounter chlorpyrifos-methyl stress. The depletion in glycogen level, in the present investigation, may also be due to direct action of chlorpyrifos-methyl on oxidative phosphorylation as observed in case of *P. americana* following treatment with lindane[27]. The observed enhancement in reducing sugar level, in the present investigation, may be due to gluconeogenesis and /or decreased sugar utilization as has been reported in case of *C. cephalonica* following exposure of malathion[54,55,56] and dimethoate[56].

Methoprene caused a significantly dose-dependent ($p < 0.05$) decrease in glycogen level and a significantly dose-dependent ($p < 0.05$) enhancement in reducing sugar level in the haemolymph as well as in the fat body of the larva of *E. cautella*[18]. A significant reduction in glycogen levels was observed in insect *Choristoneura fumiferana* following exposure to methoprene[42]. Pyriproxyfen exposure also caused a significant decrease in glycogen reserve in haemolymph and fat body tissues of adult desert locust, *Schistocerca gregaria*[29,52]. Data obtained in the present investigation indicate that the

mixture of chlorpyrifos-methyl + methoprene (9:1) ratio caused a significantly dose-dependent ($p < 0.05$) decrease in glycogen level and a significantly dose-dependent ($p < 0.05$) enhancement in reducing sugar level in hemolymph as well as fat body tissues of the larva of *E. cautella* (Table 1,2). This finding is in accordance with Shakoori and Saleem[49] who observed a significant decrease in glycogen reserve and a significant enhancement in reducing sugar content in the larva of *T. castaneum* following exposure of a mixture of permethrin (200 ppm) and malathion (20 ppm).

Acid phosphatase plays a significant role in catabolism, pathological necrosis, autolysis and phagocytosis[25,11,2]. It also helps in energy liberating processes[24]. Alkaline phosphatase has been reported to be involved in the transport of metabolites across the cell membranes[64], secretory activity[36] and hydrolysis of phosphomonoesters under the alkaline condition[41].

Earlier studies revealed that sublethal concentrations of Chlorpyrifos-methyl and methoprene caused a significantly dose-dependent ($p < 0.05$) enhancement in the activity of acid phosphatase and a significantly dose-dependent ($p < 0.05$) reduction in the activity of alkaline phosphatase in the haemolymph and fat body tissues of the almond moth larvae[18]. Pyriproxifen induced significant enhancement in the activity of acid phosphatase in the heamolymph and fat body tissues of sun pest, *Eurygaster integriceps*[67] but contrary to this, it caused a significant reduction in the activity of this enzyme in case of third instar larvae of the elm leaf beetle, *Xanthogaleruca luteola*[62], both are beetles of different genera but the same compound acts in a different way. Thus, it is clear that JHAs are species specific. Similarly, JHAs induced increase in acid phosphatase activity have also been reported in various insects such as pyriproxyfen on *C. pipiens*[28] , pyriproxyfen on *P. gossypiella* and *E. insulana*[5] and on *A. ipsilon*[31]. Our present results are supported by the findings of pyriproxyfen treated *C. pipiens*[28], *P. gossypiella* and *E. insulana*[5], *A. ipsilon*[31] *E. integriceps*[6].

Present data on phosphatase activity reveals that all the three sublethal concentrations of the mixture of chlorpyrifos-methyl + methoprene (9:1 ratio) caused a significantly dose-dependent ($p < 0.05$) enhancement in acid phosphatase activity (Table 3) and contrary to this, a significantly dose-dependent ($p < 0.05$) reduction in alkaline phosphatase activity (Table 4) in both the tissues of the larva of this moth. Similar results were observed in the larvae of *T. castaneum* following treatment with a mixture of permethrin and malathion[49]. In addition, a significant increase in acid phosphatase activity and a significant decrease in the activity of alka-line phosphatase was also reported in the larvae of *S. littoralis*[30] when exposed to chlorosan- a mixture chloropyrifos 24% + cypermethrin 5%. They also reported that

when larvae of *S. littoralis*[30] were exposed to engeo- a mixture of thiamethoxam 14.1% + lambda-cyhalothrin 10.6% a significantly higher increase in acid phosphatase activity occurred in comparison to the exposure of chlorosan.

On the basis of the present investigation it may be concluded that synergistic mixture of chlorpyrifos-methyl + methoprene in the ratio of 9:1 perturbes the metabolic flux of the larva along with other biochemical parameters that affects its growth, development and contributes to lethal action of this synergist. It deserves mention that synergistic application of these insecticides of different groups having different mode of action might ofcourse meet the problem of development of insecticide resistance in almond moth, *E.Cautella* in particular and pest population in general.

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