Ames test evaluation of mutagenicity of some Thai grilled pork and repeatedly heated oil extracts

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Submission: 15 July 2020 Revised: 18 November 2020 Accepted: 26 January 2021

ABSTRACT

The purpose of this study is to investigate the mutagenicity potential of some grilled pork, pork dripping and repeatedly heated oil extracts using the Ames test. These included grilled pork, pork dripping, heated oil of batter-fried chicken, heated oil of fried chicken, heated oil of fried fish-bar and heated oil of fried mackerel collecting from Thai local markets. The mutagenic activities of all sample extracts were examined using *Salmonella typhimurium* strains TA98 and TA100 in absence of a metabolic activation system (reaction with/without nitrite salt) under acidic conditions. The extracts of the samples were added into the plate culture at concentrations of 0.08, 0.16, 0.40 and 0.80 mg extract/plate to evaluate the correlation between the concentration of the extract and the number of bacterial colonies. Results indicated that none of the sample extracts showed mutagenic activity without nitrite treatment. After nitrite treatment under acidic conditions, all pork dripping samples showed direct-mutagenic activity towards *S. typhimurium* TA98 and TA100 at a concentration of 0.80 mg/plate, and all grilled pork samples exhibited weak mutagenicity only with TA98 at a concentration of 0.80 mg/plate. The repeatedly heated oil extracts showed no mutagenicity with TA98 or TA100. The mutagenic activity was found only at the maximum dose tested, so the sample dose-response relationship was not seen. Only the pork dripping from the market D sample was evident that the sample was mutagenic in both substitutions: frameshift and base-pair. However, minimizing the consumption of grilled and fried food has to be recommended.

Keywords: Grilled pork, mutagenicity, nitrosation, *Salmonella typhimurium*, frying oil

Thai J. Agric. Sci. (2020) Vol. 53(4): 218−227

INTRODUCTION

Nowadays, grilled and fried foods are popular among most people due to their unique flavor, convenience and affordability. Recent research has indicated that eating grilled and fried food can be harmful to human health by increasing the risk of developing cancer. When the meat is cooked using high temperatures, muscle proteins are altered. The reaction is the formation of cancer-causing compounds called polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HCAs) and N-nitroso compounds (NOCs) which cause DNA damage only after they are metabolized by cytochrome P450 enzymes in the human body and increase the incidence of certain cancers, especially colon and stomach cancer (Loh *et al*., 2011; Kobayashi, 2018). Over time, the high consumption of these grilled or fried foods may result in a high risk of cancer. There is sufficient evidence that those compounds in overcooked meat might be carcinogenic. Sugimura *et al*. (2004) reported that exposure to HCAs can cause cancer in many organs in rodent models, including the colon, breast and prostate. Loh *et al*. (2011) reported that dietary NOCs were associated with the incidence of rectal cancer. The richest sources of N-nitroso were found in sausage, bacon and smoked meats. Dietary NO could stimulate carcinogens in 40 animal species, including humans (Stuff *et al*., 2009). In addition, NOCs increased the incidence of developing pancreatic cancer risk in humans (Risch, 2003; Zheng *et al*., 2018). NOCs can also be generated endogenously from the intake of nitrate and nitrite compounds, which can react with the degradation products of amino acids in the stomach.

The use of food additives in meat products is essential for color formation, antimicrobial, flavor-enhancing and reducing food alteration. The most widespread food preservatives used in meat products are sodium nitrite, sodium nitrate, potassium nitrite and potassium nitrate. Sodium nitrite is also present in green leafy vegetables (505–1,573.4 mg/kg), especially leafy vegetables such as kale, lettuce, bok choy, morning glory and chives. Vegetables grown by hydroponic method had an average of 1,729 mg/kg of sodium nitrate and the highest nitrite was 4,529.23 mg/kg, which is much higher than the standard level. It is considered that the leafy vegetables and petioles are the food groups which is the source of the intake nitrates and nitrites to the body (Phupaibun *et al*., 2009). Sodium nitrite is mutagenic in *S. typhimurium* TA1530, TA1535 and TA100 and has clastogenicity in culture mammal cells (Matsumoto *et al*., 2008).

The Ames test is one of the most widely employed protocols that has been recognized as a valid test for accessing the mutagenic potential of chemical compounds in foods and consumer products due to its simplicity, sensitivity, relatively low cost and flexibility to different experimental settings (Ames and McCann, 1981). This test was employed to detect the induction of frameshift mutations and base–pair substitutions using specific bacterial strains. The mutagenicity of the substances was identified by the following principles: 1) The extracted sample concentrations and the growth of the colonies had to show a dose-response relationship, i.e., as the extracted sample concentration increased, the mutant colony increased 2) The number of mutant colonies (affected by extracting sample) had to be higher than the number of mutant colonies (spontaneous mutation) in at least two extracted sample concentrations and 3) the number of mutations (affected by extracting sample) had to be twice as high as the spontaneous mutations in at least one extracted sample concentration (Tejs, 2008; Hakura, 2014).

Previous research has shown that 50–70% of known carcinogens may be determined by this protocol (Gautam *et al*., 2016). The Ames test allows for testing of the mutagen content of food products. For instance, Krone and Iwaoka (1987) reported that canned beef broth, chili, hash, roast beef, pink and red salmon, and mackerel contain substances that exhibiting mutagenicity, which was 20 times higher mutagenic than spontaneous revertant colonies in the Ames test.

Research has indicated that the consumption of vegetable oils as a result of a prolonged frying process can induce many forms of cancer (Khalil *et al*., 2009). Fong *et al*. (1980) reported that samples of peanut oil extracts from a local market in Hong Kong posed mutation ability to *S. typhimurium* (TA98 and TA100). Nevertheless, two samples of corn oil extract possessed no mutagenic effect in the *S. typhimurium* colony. Abeer *et al.* (2014) discovered that the tumor suppressor *p53* gene mutations were induced by repeated fried palm oil in rat liver because of the formation of the oxidative compound of heterocyclic aromatic amines (HAAs) in repeated fried oils which could produce free radicals causing DNA mutation in animal tests. These substances may also have an effect on mutagenicity in bacterial colonies. According to Asare *et al*. (2013), high-energy red palm oil has mutagenic activity toward both strains of *S. typhimurium* TA98 and TA100, and this effect increased with increasing heating frequency.

Some individuals with specific genetic variants were more susceptible to the cancer risk associated with processed meat consumption. However, more research will need to be done to find a link between processed meat consumption and cancer incidence. The purpose of this study was to present more evidence exploring the connection between processed meat, dripping from grilled meat and repeatedly heated oil extracts and carcinogenicity by using the Ames test. The research presented here was targeted to corroborate the possibility that the mutagenicity detected in the local food processing may represent a risk of cancer in people.

MATERIALS AND METHODS

Samples Preparation and Extraction

Samples were randomly gathered from four markets (A, B, C and D) sites viz. Ngam Wong Wan road (market A and B) and Phaholyothin road (market C and D), located in Bangkok, Thailand. The samples included Thai grilled pork, pork dripping, heated oil of batter-fried chicken, heated oil of fried chicken, heated oil of fried fish-bar and heated oil of fried mackerel. The rice bran oil and palm oil were the oil that sellers used. The experiments were conducted at the Institute of Food Research and Product Development (IFRPD), Kasetsart University, Bangkok, Thailand.

The grilled pork sample was chopped and blended before weighing while the pork dripping and heated fried oil samples were weighed directly. Fifty grams of each sample was blended with 1:1 (v/v) methanol-chloroform at room temperature. The mixture was shaken for 5 hr and stored at –20°C for 24 hr. Then, the mixture was filtered through Whatman No.1 filter paper. The filtrate was evaporated at 45°C under low pressure using a rotary evaporator (Rota vapor R–200, Buchi, Switzerland). The crude extract was kept at –20°C for the next experiment.

One gram of the crude extract was added into 5 mL of 70% ethanol and the pH was adjusted to 2 by 0.2 N HCl, then mixed with 5 mL petroleum ether and allowed to stand for 5 hr. The petroleum ether phase was discarded and the ethanol phase was collected in a 250 mL flask. This process was repeated three times. After the pH of the ethanol layer was adjusted to 10–12 by 0.1 N NaOH, an equal volume of dichloromethane was added, mixed, and the solution was allowed to stand for 5 hr. This process was also repeated three times. The dichloromethane was evaporated from the solution until the crude extract was derived. The crude extract was then redissolved in DMSO to a concentration of 0.1 g/mL and kept at –20°C (Saengprakai *et al.,* 2015).

Microbial Inoculation

S. typhimurium (TA98 and TA100) was inoculated in nutrient broth according to the procedure developed by Kangsadalampai *et al*. (1996). Each culture (10 µL) was pipetted into the flask containing the nutrient broth (No.2, Oxoid Ltd, Basingstoke, UK, analytical grade, 99%). The flasks were kept in an incubator at 37°C for 16 hr.

Mutagenic Activity Assay with Nitrite Treatment

Crude extract,100 µL, and 650 µL of 0.2N HCl were added in a test tube. The pH of the solution remained in the range of 3.0–3.5, then 250 µL of 2M

sodium nitrate (BDH Chemicals, Poole, England, analytical grade, 99%) was added to the reaction mixture. The final dose of nitrite was 500 mM. The reaction tubes were placed in a shaking water bath at 37°C for 4 hr. After that, the reaction was stopped by soaking in an ice-bath for 1 min. Two hundred and fifty microliters of 2M ammonium sulfamate were added to the test tube in order to decompose the residual nitrite and allowed to rest for 10 min in an ice-bath.

Mixed nitrite treated mixture (10, 20, 50 and 100 µL) with 890, 880, 850 and 800 µL of NaPO4–KCl buffer (pH 7.4), added 100 µL of each tester strain and incubated at 37°C in shaking water bath for 20 min. After incubation, added 2 mL of top agar containing 0.5 mM L-histidine and 0.5 mM biotin, mixed well, and poured onto a minimal glucose agar plate. The final concentrations of the extracts per plate were 0.08, 0.16, 0.40, 0.80 mg, respectively. The plate was incubated at 37°C in darkness for 72 hr.

Mutagenic Activity Assay without Nitrite Treatment

The assay was done as described above. The volume of sodium nitrite (250 µL) and ammonium sulfamate (250 µL) were replaced by sterilized distilled water.

Negative and Positive Control

The DMSO was used as a negative control to determine spontaneous reversion. The 1–aminopyrine (1–AP) treated with nitrite in acid solution, products formed of nitroso-compounds and direct-acting mutagen product, was used as a positive control.

Data Analysis

The number of histidine (His^+) revertant colonies were manually counted. Toxicity or killing effect was assessed by investigation of the background bacterial lawn (thinning or presence of microcolonies) using an inverted microscope. The mutagenicity of sample extract was expressed as mean ± standard deviation (SD) of four replications. Samples exhibiting mutagenicity higher than twice spontaneous revertants and also a concentration-response relationship were considered to be mutagenic.

RESULTS AND DISCUSSION

The mutagenic effects of the grilled meat extracts on *S. typhimurium* (TA98 and TA100) are given in Table 1. It was found that with nitrite salt added, the grilled meat extracted a sample from market A and market B (at 0.80 mg/plate), market C (at 0.40 and 0.80 mg/plate) and D (at 0.40 mg/plate) showed a mutation effect on *S. typhimurium* TA98. The partial killing effect was observed at a high dose (0.80 mg/plate). Since microcolonies were found on the plate when endoscopy with an inverted microscope, the resulting toxicity was expected from grilled pork from market D extracts treated with nitrite was unlikely to be caused by DMSO toxicity due to the presence of a parallel test. The extract of grilled pork from market D treated without nitrite involved in the experiment gave normal values. However, without nitrite, no extracted samples showed any mutation effect on *S. typhimurium* TA98 and TA100.

Table 1 Mutagenicity of Thai grilled pork treated with sodium nitrite on *S. typhimurium* strains TA98 and TA100

Sample	Concentration of sample (mg/plate)	No. mutation colonies[a]/plate			
		TA98		TA100	
		Without NO^-_2	With NO^-_2	Without NO^-_2	With NO^-_2
Negative control	0.00	34 ± 1	33 ± 3	74 ± 4	77 ± 6
Positive control[b]	0.00 µg/plate	–	30 ± 2	–	79 ± 4
	0.06 µg/plate	–	149 ± 24	–	216 ± 18
	0.12 µg/plate	–	382 ± 22	–	332 ± 21
	0.24 µg/plate	–	990 ± 47	–	511 ± 14
Thai grilled pork from market A	0.08	20 ± 1	33 ± 2	91 ± 2	94 ± 11
	0.16	23 ± 1	37 ± 3	108 ± 14	96 ± 2
	0.40	24 ± 6	47 ± 1	112 ± 9	112 ± 21
	0.80	31 ± 2	***76 ± 3***	118 ± 2	106 ± 16
Thai grilled pork from market B	0.08	35 ± 4	31 ± 2	83 ± 6	108 ± 12
	0.16	39 ± 2	37 ± 4	74 ± 12	107 ± 11
	0.40	44 ± 3	41 ± 1	74 ± 10	132 ± 13
	0.80	46 ± 4	***85 ± 4***	109 ± 6	146 ± 5
Thai grilled pork from market C	0.08	31 ± 5	32 ± 4	81 ± 12	101 ± 16
	0.16	37 ± 17	53 ± 1	83 ± 8	105 ± 14
	0.40	39 ± 13	69 ± 6	87 ± 6	121 ± 10
	0.80	43 ± 2	***72 ± 0***	98 ± 2	129 ± 13
Thai grilled pork from market D	0.08	35 ± 1	42 ± 1	77 ± 4	73 ± 4
	0.16	37 ± 1	56 ± 15	78 ± 3	78 ± 4
	0.40	40 ± 1	***74 ± 5***	81 ± 7	102 ± 9
	0.80	45 ± 3	PK	92 ± 10	119 ± 12

Note: [a] mean (from 4 replicates) ± standard deviation, [b] 1–aminopyrene, PK = partial killing, number of mutation colonies in bold and italic type means number of revertant doubles the spontaneous yields

The mutation results of the pork dripping extracts with *S. typhimurium* (TA98 and TA100) were obtained (Table 2). Extracts from markets A, B and C (at 0.80 mg/plate) had mutagenicity toward *S. typhimurium* TA98 and TA100 with nitrite reaction. Only the pork dripping extracts from market D showed mutagenicity on *S. typhimurium* TA98 and TA100 at concentrations of 0.40 and 0.80 mg/plate. However, after being reacted without nitrite solution under acidic conditions (pH 3–3.5) for 4 hr, no extracted samples showed any mutagenicity effect toward *S. typhimurium* TA98 and TA100. The mutation results of *S. typhimurium* (TA98 and TA100) on the heated oil from batter-fried chicken, fried chicken, fried fish-bar, and fried mackerel is included in Table 3. The results revealed that no extracted samples showed any mutation effect on *S. typhimurium* (TA98 and TA 100).

Table 2 Mutagenicity of Thai pork dripping treated with sodium nitrite on *S. typhimurium* strains TA98 and TA100

Sample	Concentration of sample (mg/plate)	No. mutation colonies[a]/plate			
		TA98		TA100	
		Without NO^-_2	With NO^-_2	Without NO^-_2	With NO^-_2
Negative control	0.00	34 ± 1	33 ± 3	74 ± 4	77 ± 6
Positive control[b]	0.00 µg/plate	–	30 ± 2	–	79 ± 4
	0.06 µg/plate	–	149 ± 24	–	216 ± 18
	0.12 µg/plate	–	382 ± 22	–	332 ± 21
	0.24 µg/plate	–	990 ± 47	–	511 ± 14
Pork dripping from market A	0.08	38 ± 1	53 ± 3	69 ± 7	76 ± 6
	0.16	55 ± 5	61 ± 6	80 ± 4	81 ± 7
	0.40	42 ± 1	55 ± 3	86 ± 19	73 ± 0
	0.80	51 ± 6	***92 ± 5***	88 ± 13	***182 ± 8***
Pork dripping from market B	0.08	49 ± 11	46 ± 4	74 ± 1	54 ± 0
	0.16	44 ± 1	52 ± 0	75 ± 5	77 ± 12
	0.40	54 ± 11	57 ± 13	73 ± 12	76 ± 9
	0.80	57 ± 7	***90 ± 4***	76 ± 24	***195 ± 7***
Pork dripping from market C	0.08	50 ± 16	40 ± 10	82 ± 27	99 ± 30
	0.16	48 ± 2	55 ± 6	83 ± 10	108 ± 5
	0.40	45 ± 1	60 ± 6	93 ± 4	111 ± 10
	0.80	48 ± 6	***84 ± 2***	101 ± 11	***166 ± 17***
Pork dripping from market D	0.08	50 ± 16	39 ± 9	77 ± 6	97 ± 8
	0.16	38 ± 7	60 ±14	85 ± 9	139 ± 9
	0.40	22 ± 14	***121 ± 21***	96 ± 8	***186 ± 43***
	0.80	14 ± 20	***143 ± 16***	91 ± 2	***196 ± 61***

Note: [a] mean (from 4 replicates) ± standard deviation, [b] 1–aminopyrane, number of mutation colonies in bold and italic type means number of revertant doubles the spontaneous yields

The results revealed that after adding nitrite solution into the grilled pork extract solution, the extracts of grilled pork from all markets showed mutation results on *S. typhimurium* TA98 without an enzyme-activating system. This may have been because the sample extracts react with nitrite under acidic conditions and this generates mutagenic nitroso and nitrosamine compounds.

The extract of the grilled pork was the "frameshift mutation" type but did not show mutagenicity toward *S. typhimurium* TA100. This study was supported by the experiment of Koutros *et al.* (2008) who observed high-temperature meat cooking and found substances such as PAHs, HCAs and NOCs after reacting with nitrite under acidic conditions without an activating system and

that they were capable of causing mutation toward both types of *S. typhimurium* (TA98 and TA100).

With nitrite addition, the extracts derived from the pork dripping (from markets A, B, C and D) produced mutation colonies on both types of *S. typhimurium* TA98 and TA100. The positive test indicated that treatment of the pork dripping with nitrite for 4 hr at 37°C under acid solution may produce some direct mutagens which might belong to nitroso compounds.

Table 3 Mutagencity of repeatedly heated oil treated with sodium nitrite on *S. typhimurium* strains TA98 and TA100

Sample	Concentration of sample (mg/plate)	No. mutation colonies[a]/plate			
		TA98		TA100	
		Without NO^-_2	With NO^-_2	Without NO^-_2	With NO^-_2
Negative control	0.00	34 ± 1	33 ± 3	74 ± 7	77 ± 6
Positive control[b]	0.00 µg/plate	–	30 ± 2	–	79 ± 4
	0.06 µg/plate	–	149 ± 24	–	216 ± 18
	0.12 µg/plate	–	382 ± 22	–	332 ± 21
	0.24 µg/plate	–	990 ± 47	–	511 ± 14
Heated oil of fried chicken	0.08	33 ± 1	31 ± 1	78 ± 8	84 ± 4
	0.16	36 ± 2	34 ± 6	87 ± 5	91 ± 2
	0.40	40 ± 1	36 ± 4	89 ± 7	95 ± 3
	0.80	44 ± 4	38 ± 5	94 ± 2	106 ± 8
Heated oil of batter-fried chicken	0.08	35 ± 7	48 ± 2	65 ± 17	78 ± 9
	0.16	36 ± 1	45 ± 4	67 ± 3	85 ± 6
	0.40	43 ± 3	53 ± 3	80 ± 9	93 ± 2
	0.80	53 ± 7	55 ± 4	106 ± 8	104 ± 4
Heated oil of fried fish–bar	0.08	32 ± 4	25 ±1	65 ± 12	80 ± 3
	0.16	39 ± 1	33 ± 1	71 ± 13	82 ± 4
	0.40	42 ± 3	36 ± 4	83 ± 7	83 ± 3
	0.80	50 ± 5	46 ± 6	97 ± 9	89 ± 1
Heated oil of fried mackerel	0.08	32 ± 4	34 ± 5	74 ± 4	63 ± 6
	0.16	39 ± 12	49 ± 5	80 ± 1	76 ± 10
	0.40	45 ± 4	50 ± 10	87 ± 17	81 ± 6
	0.80	50 ± 1	58 ± 7	92 ± 2	96 ± 6

Note: [a] mean (from 4 replicates) ± standard deviation, [b] 1–aminopyrene, number of mutation colonies in bold and italic type means number of revertant doubles the spontaneous yields

Kangsadalampai *et al.* (1996) sampled various types of grilled meats, including grilled sea bass, smoked fish, grilled catfish, roasted chicken wings, grilled lean pork and Isan sausage. The sample extract was reacted with nitrites and tested for mutagenic activity. All grilled meats exhibited frameshift mutation and base-pair substitute mutation at concentrations in the range of 0.2–0.4 mg/plate and were demonstrated with 15 types of standard PAHs mutagen. The results showed that all PAHs reacted with nitrites were identical to those of the roasted food samples. PAHs showed mutagenic activity in a dose-response relationship which was expected to be converted to nitro-PAHs. Besides, Kitamura *et al.* (2006) found that HCAs contaminated with grilled food, when treated with nitrites, formed nitric oxide-mediated nitrosation products to the point of forming compounds. The N-nitroso-HCA and nitro-HCA are made up of HCA group compounds. There are many types of nitrites transformed by nitrites, such as IQ, MeIQ, MeIQx, and PhIP. These transformed substances become harmful substances that can damage DNA and cause cancer, such as the formation of HCA-DNA adducts which were found in human colon cancer cells. High heat-cooked meats contain large amounts of HCAs. Therefore, it is possible that the mutagenic compounds in this research are the same group.

Moreover, eating meats cooked at high temperatures or direct grilling meat over an open flame produces chemicals called HCAs which are on the list of cancer-causing agents. This result corroborates Yano *et al*. (1988) who discovered that the processed chicken, pork and beef were mutagenic to both types of *S. typhimurium* (TA98 and TA100) after nitrite treatment. In contrast, unprocessed meat did not produce any mutation effect on these bacteria after nitrite treatment.

The results of the Ames tests on the grilled meat and the pork dripping extracts revealed that the mutagenicity of the extract samples was affected by the concentration levels of the extract samples. As the concentration increased, the extract samples exhibited stronger mutagenicity. According to Kitano *et al*. (2002), a dose-dependent mutagenic activity was found when the mixture of potassium sorbate, ascorbic acid and ferrous salt was applied to *S. typhimurium* TA100 using an Ames test without S–9 mix.

The highest dose, 0.80 mg/plate, of the extract of grilled pork from market D had a partial killing effect after treated with nitrite. This result was supported by the experiment reported by Ovrevik *et al*. (2009) that found many nitro-PAHs can damage cellular constituents, resulting in either acute toxicity or genotoxicity, lead to cell death since the DNA damage is too severe to be repaired.

From the experimental results, heated oil of batter-fried chicken, fried chicken, fried fish-bar and fried mackerel did not show any mutagenicity effects toward the bacterial colonies with or without nitrite reaction. These may result from the heating process is different from grilling, which is the direct heating from heat-origin to meat. The direct heating from grilling is inducing a variety of mutagens in meat. Iwasaki *et al*. (2010) found that the popular mutagen contaminants (MeIQx, 4,8–DiMeIQx and PhIP) in fried meat are lower than grilled meat significant dramatically. Furthermore, there are several previous reports suggested that palm oil and rice bran oil contain antioxidant compounds, tocopherols and tocotrienols. Therefore, these compounds could result in the oxidative stability of the oil and might affect the mutagen formation in deep-fried meat (Kusum *et al*., 2011; Tsouko *et al*., 2019).

CONCLUSIONS

Extracts from the grilled pork and pork dripping samples could produce direct-acting mutagens after nitrosation. The repeatedly heated oil extracts use for fried food showed no mutagenicity against both strains (TA98 or TA100) with or without nitrite salt. Nevertheless, the researchers recommend that the consumption of grilled and fried foods should be minimized and it is also recommended that the processing of any meat should avoid charring or

grilling of the meat or food using a high temperature because the way the food is processed is a factor that contributes to increased cancer incidence.

In a future study, analysis of the type and amount of mutagenic contaminants in grilled food may be studied, together with the somatic mutation and recombination test, a model studied in the Drosophila, classified as Eukaryotes with a toxin-altering enzyme system that is similar to humans and has similar cellular and chromosomal characteristics to humans.

ACKNOWLEDGEMENTS

This work was supported by the Center for Advanced Studies in Tropical Natural Resource, Kasetsart University, Bangkok, Thailand and the Research Unit of the Orchid Tissue Culture, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom, Thailand.

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