



Relative toxicity of Lead and Nickel on acid and alkaline phosphatase in epigeic earthworm *Eisenia fetida* (Savigny, 1826)

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ARTICLE INFO

Received: 05.10.2020;
Revised: 02.01.2021;
Accepted: 10.01.2021

Keywords: Lead, Nickel, *Eisenia fetida*, acid phosphatase (ACP), alkaline phosphatase (ALP).

ABSTRACT

Among the soil fauna, earthworms are not only acting as bioindicator to determine soil pollution but also they provided with some specific enzymatic biomarker to decode the soil contamination. In the present heavy metal toxicity study, the LC50 of lead (Pb) and nickel (Ni) were determined in both artificial and natural ground soil by acute toxicity test (14 days) in *Eisenia fetida*. Low observed effective concentration (LOEC) of mixture of both metals (Pb and Ni) were also determined through repetitive experimental acute toxicity test. In the chronic toxicity test (28 days), the experimental set up had been arranged as control (C), T1(1506.25 mg Pb), T2 (3012.5mg Pb), T3 (193.75mg Ni), T4 (387.5mg Ni), T5 (753.125mg Pb and 96.875mg Ni) and T6(1506.25mg Pb and 193.75mg Ni) per Kg of dry soil. After end of chronic periods, specific activity of acid and alkaline phosphatase were determined in the earthworm tissue. The mean difference of recorded specific activity values of both enzymes were significant ($P < 0.05$) and also showed a significant negative correlation ($P < 0.05$) between the specific activity both enzymes.

1. Introduction

Several kinds of anthropogenic activity and use of agrochemicals greatly affects the normal ecological health of soil that is correlated with physiological health of terrestrial biome. Heavy metal pollution is an ecophysiological threat to paedo-fauna that increases in now days. This type of pollution of soil is responsible for

beginning of bioaccumulations followed by biomagnifications in terrestrial ecosystem. In the last decades, soil pollution has been enhanced enormously due to industrial activities, urban sewage, intensive use of biocides and chemical fertilizers. Diffusible heavy metals contamination occurs in soil from variable sources, such as agricultural field,

industrial wastes, mining and sludge residues (Wuana et al., 2011). Mandal and Sengupta (2002) reported that the coal fly ash contains heavy and trace metals like arsenic, beryllium, cadmium, chromium, copper, cobalt, nickel (Ni), lead (Pb) etc. Heavy metals released from coal-fired thermal power station and deposited in soil, significantly responsible for environmental degradation of surrounding areas (Khillare et al., 2012). Heavy metals do not decompose or disappear from soil although their release to the ecosystem can be controlled by specific way of treatment (Brusseau, 1997). The sources of metal pollution include natural sources (Hobbelen et al., 2006), agricultural activities, municipal, industrial waste, sewage sludge etc (Salehi and Tabari, 2008), and also comes from particulates from aerial deposition of vehicular emission (Ward and Savage, 1994). The heavy metals are diffused in the soil rapidly (Lanno et al., 2004). Previous studies exhibited that earthworms accumulate metals, such as Cd, Cr, Cu, Co, Ni, Pb, Zn from soil under both field and laboratory conditions (Li et al., 2010). Kammenga et al. (2000) reported that earthworms acts as good sentinel organisms of soil pollution because they contact with soil pore water directly. In the environment, earthworms are frequently exposed to heavy metals and so their efficient detoxification systems maintain certain molecules that acts as molecular biomarkers

of soil contamination. The earthworm acts as good bioindicator in terrestrial ecosystem due to interest of pollution (Suthar et al. 2008). Different kinds of soil bioindicator and biochemical indicator has been worked out in soil fauna by many researcher. Enzymes are called as biological catalysts that catalyzed several reactions on its substrate molecule to produce product, in association with specific specificity and kinetics under suitable environmental (external and internal) parameters. Biochemically, the enzymes are simple or complex protein molecules produced in the living cells where they were performing various physiological reactions. The assessment of any the environmental effect of a pollutant on mortality test of animal was a colloquial measure. However, sublethal effects of heavy metals acts as gradual and indicative physiological modification can be found as detrimental as mortality to the animal's survivability (Sonawane, 2017). Quantitative assessment of biocatalyst is a plausible indicator of environmental pollution such as heavy metal contamination that affect physiological and biochemical condition of organisms. Physiological activity of many enzymes including lysosomal hydrolytic enzymes are inhibited by heavy metals (Cheng, 1983). Lysosome contains different kinds of acid hydrolases are responsible for intracellular digestion (Berhet, 1965; de Durve and

Wattiaux 1966). The distribution of both acid phosphatase (ACP) (EC 3.1.3.2) and alkaline phosphatase (ALP) (EC 3.1.3.1) are differing in the cellular and sub cellular localization. Lysosomal enzyme, ACP catalyzes the phosphomonoester substrates in acidic condition pH, 4.8. ALP is multifunctional enzyme, catalyzes phosphomonoester substrates in alkaline medium pH, 9.8 (Bhargavan, 2010; Sadasivam and Manickam, 2015). The specific activity of acid phosphatase and alkaline phosphatase is a biochemical indicator during hyperactivity of lysosomal digestion process that also involving with the metallic stress (Bhargavan, 2010). ACP is an important lysosomal marker enzyme and ALP is frequently occurs in the fraction containing plasmalemma. These enzymes are associated with the cell differentiation and growth of organisms. (Ram and Sathyanesan, 1985). Bhattacharya et al. (1975) and Anees (1976) reported that the activity of ACP and ALP enzymes being changed with the treatment of endrin and heavy metals like Cu, Hg and Zn on fish *Clarius batrachus* and in the fish *Channa punctatus* respectively. The enzymatic efficiency become changed by metallic exposure in some way associated with the response of physical and physiological parameters, co-factors, and also its Michaelis-Menten constant (K_m) (Jackim, 1974). The inhibition of acid phosphatase activity and the

acceleration of the alkaline phosphatase activity were found after fungicide exposure (Chakravorty et. al., 2017). Ukpabi et al. (2013) reported that the activity of ALP and ACP could be used as biomarkers for detection of cellular damage after the treatment of metal contaminated fertilizer in *E. fetida* or for soil contamination surveys.

This experiment a attempt to study the effect of selected heavy metals on the activity of acid phosphatase and alkaline phosphatase. The present work has been done for evaluating the LC50 of Lead and Nickel in acute toxicity followed by chronic toxicity that determine the specific activity of acid phosphatase and alkaline phosphatase in *Eisenia fetid* after exposure with sublethal doses of lead, nickel and their combination of lowest observed effective concentration (LOEC) value.

2. Materials and methods

2.1. Sample collection and culture

The sample specimens (*Eisenia fetida*, red wiggler worm) was collected from WBCADC (West Bengal Comprehensive Area Development Corporation) Tamluk Project that is regulated under Panchayet & Rural Development Department, Govt. of West Bengal, India. The specimens has been cultured in vermicomposting pit in Raja Narendra Lal Khan Women's College, Gope Palace, P.O- Vidyasagar University, Dist: Paschim Midnapore, Pin: 721102. The vermiculture

unit located in natural environment with shadow shade area. The vermicomposting cum cultural pit had been covered by fine meshed iron net to avoid unwanted contamination. The substrate medium consists of pesticide free fine ground soil with dried cow dung manure. Finely grinded soil particles was mixed with cow farmyard manure as 1:1 ratio and was used as the culture medium for the specimens (Ismail, 1997). The hand sorted cocoons became separated and cultured in a separate pots, those were used as experimental specimens later. The refined water has been used for maintaining moisture. The dust cow dung manure has been utilized as food for the growing test specimen and gave at a specific interval of time. The compost use as biofertilizer in the flower garden of this college. Chakravorty et al., (2017) pointed out that *Eisenia fetida* are easily breed and culture at the Environmental Test Chamber in laboratory with different kinds of organic medium. Therefore, the species is very scientifically specific for ecophysiological and toxicological studies.

2.2. Acute toxicity test

Adult age synchronized *Eisenia fetida* worms (with visible clitellum) were blotted on filter paper and weighed individually. The worms were acclimated for 24 hrs after removal from the mother culture prior to experimental use, washed with redistilled water and then hold on

wet filter paper in the dark environmental chamber at desirable temperature ($28^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$) and humidity (60-65%). This process was allowed to defecation of gut contents (Rathi et al., 2011). Acclimated *Eisenia fetida* was used for the acute toxicity test in both artificial soil and natural ground soil medium. Age synchronized individual worm weighing about 270-290 mg was selected for exposure in this test. Experimental culture of specimens were done in inert polythene boxes (16 X 12 X 1 cm, total area, 192 cm²). The artificial soil was comprised (by dry weight) of 70% quartz sand, 20% kaolinite, and 10% finely sieved paddy husk, with the pH adjusted to 6.0 ± 0.5 by the addition of calcium carbonate (CaCO_3) (De Silva et al., 2009; Sanyal et al., 2015). The LC50 of lead (Pb) and nickel (Ni) was also determined in the ground soil. The LC50 of lead (Pb) and nickel (Ni) were determined in both artificial and natural ground soil as per acute toxicity test, OECD guideline 207. From pesticide free grassland, the collected soil samples were sun-dried, grinded and sieved as particle size of 0.25 mm that acts as the test medium or substrate. Individually Lead acetate trihydrate $[(\text{CH}_3\text{COO})_2\text{Pb} \cdot 3\text{H}_2\text{O}]$ and Nickel acetate tetrahydrate $[(\text{CH}_3\text{COO})_2\text{Ni} \cdot 4\text{H}_2\text{O}]$ are used for contamination of the experimental soil. Three replicates was kept up for each set of experiments together with control set simultaneously. The experimental inert boxes

were remained as undisturbed for 48 hours before inoculation of worms for softening of medium and thermo-stabilization. Defecated and acclimated earthworms (10 pieces per box) were inoculated in each experimental boxes. The experimental set up was performed in an Environmental Test Chamber at a constant temperature of $28 \pm 0.5^{\circ}\text{C}$ and 60-65% relative humidity. The entire experiment was performed for three times (Dasgupta et al., 2010).

The physiochemical parameters of both artificial soil and natural soil such as Organic carbon Content, moisture content, and pH were determined in constant room temperature and moisture content. Infrared Torsion balance moisture meter utilized for determination of moisture content of the soil (Chakravorty, 1990). The pH and organic carbon content of both soil were determined by the method of Piper (1942) in stable temperature and moisture. Those specimens showed no observable evidence of life after every 7 days of interval, even when poked with a blunt needle, were considered as dead and were removed from the box due to avoid unwanted contamination. The soil was checked at specific regular interval (weekly) for detection of moisture loss by weighing the test containers and replenished with redistilled water if required. After end of the study (14 days), the mortality were assessed by EPA probit analysis

program 1.5 (US EPA, 2006).

2.3. Chronic toxicity test

The sublethal doses of LC50 of two selected heavy metals (Pb and Ni) were used individually and jointly for chronic toxicological study of bioaccumulation and metallothionein response in the above described specimen. The chronic toxicity test was performed in similar way as described above in acute toxicity test in ground soil for a period of 28 days of study. Very finely grinded (0.25 mm) and dried cow dung manure (5 g dry weight) was added to the test soil medium weekly to provide food for the growing worms (Chakravorty et al., 2017). Two individual sub-lethal doses of lead(Pb), nickel(Ni), and combination of lead with nickel (Pb-Ni) were applied in ground soil, along with control (C) for determining the bioconcentration factor and metallothionein response. 25% (T1 -1506.25 mg) and 50% (T2 - 3012.5 mg) of the LC50 values of the lead(Pb) and 25% (T3 – 193.75 mg) and 50% (T4 – 387.5 mg) of the LC50 values of the nickel(Ni) were applied on garden soil (Kg) for metallic exposure. The test concentrations of heavy metals combination were determined after repetitive conduction of preparatory trial experiment. The lowest observed effective concentration (LOEC) for each metals was chosen for final experimentation (Todorova et al. 2015). The mixture of both heavy metals,

lead and nickel was applied on soil (Kg) as 12.5% (T5 - 753.125 mg Pb and 96.875 mg Ni) and 25%(T6 – 1506.25 mg Pb and 193.75 mg Ni) of the LC50 values of the respective lead and nickel metals. The specimens were introduced in the experimental boxes, after that the boxes are placed in an Environmental Test Chamber and maintaining constant temperature of 28°C ($\pm 0.5^\circ\text{C}$) and 60-65% relative humidity. The earthworms were removed from the container after the end of chronic experimental period. The worms were cleanup with redistilled water followed by blotting with paper towels and then the worms were subjected for experimental findings.

Instruments Used:

Instruments	Company and Model
Electronic Balance	Mettler Toledo (New Classic MS)
Environmental Test Chamber	IIC -INSTIND
Homogenizer	Remi Electrotechnik L (Type RQP-127/A).
Centrifuge	Remi Cooling Centrifuge (C-24BL).
Spectrophotometer	Systronics (UV-VIS Spectrophotometer 11)

2.4. Determination of acid phosphatase activity (ACP)

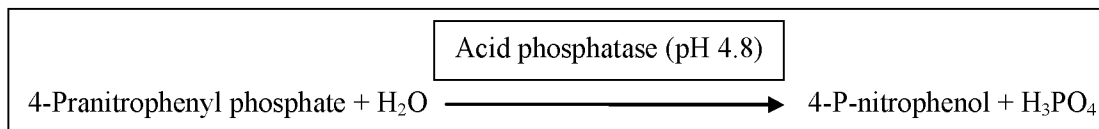
The activity of acid phosphatase was measured as described by Walter and Schutt, (1974). 250 mg fresh earthworm tissue was taken in homogenizing tube and subjected to homogenization in 5 ml normal saline. The

homogenized sample was then centrifuged at 10000 rpm for 10 minutes at normal room temperature. 0.2 ml supernatant was taken in a test tube in which 1 ml acid buffer was added and mixed thoroughly. A blank was prepared by giving 0.2 ml of 0.7% saline to 1 ml acid buffer. The test tubes were kept in the incubator for 30 minutes at 37° C. After the incubation period, 2 ml of 0.1 N NaOH solution was added to the test tubes and were mixed absolutely. The amount of liberated p-nitrophenol in tissue sample mixture gives an intense yellow colour that was measured spectrophotometrically at 405 nm wave length after adjusting the absorbance of the blank. A standard curve was drawn with the known amount of paranitrophenyl phosphate in the same procedure and the values of liberated p-nitrophenol were determined from the standard curve.

Protein concentration of each sample was quantified by the Lowry's method. A standard curve was drawn using BSA and the amount of protein in our earthworm tissue was calculated from the linear regression equation based on the standard curve (Lowry et al., 1951). The acid phosphatase activity was finally expressed in $\mu\text{g pnp/mg of protein/30 mins}$.

2.5. Determination of alkaline phosphatase activity (ALP)

The activity of alkaline phosphatase was measured as described by Walter and Schutt,

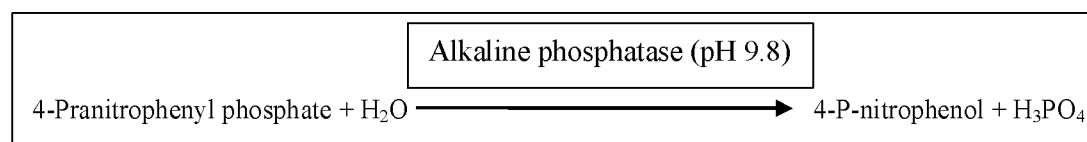


(1974). 250 mg fresh earthworm tissue was taken in homogenizing tube and subjected to homogenization in 5 ml normal saline. The homogenized sample was then centrifuged at 10000 rpm for 10 minutes at normal room temperature. 0.05 ml supernatant was taken in a test tube in which 2 ml alkaline buffer was added and mixed thoroughly. A blank was prepared by giving 0.05 ml of 0.7% saline to 2 ml alkaline buffer. The test tubes were kept in the incubator for 30 minutes at 37° C. After

curve was drawn using BSA and the amount of protein in our earthworm tissue was calculated from the linear regression equation based on the standard curve (Lowry et al., 1951). The alkaline phosphatase activity was finally expressed in µg pnp/mg of protein/30 mins.

3. Results

In our experiment, some important physicochemical parameters of both artificial and natural soil had been determined which



the incubation period, 10 ml of 0.05 N NaOH solution was added to the test tubes and were mixed absolutely. The amount of liberated p-nitrophenol in tissue sample mixture gives an intense yellow colour that was measured spectrophotometrically at 405 nm wave length after adjusting the absorbance of the blank. A standard curve was drawn with the known amount of p-nitrophenol phosphate in the same procedure and the values of liberated p-nitrophenol were determined from the standard curve.

Protein concentration of each sample was quantified by the Lowry's method. A standard

are given in table 1. The pH and organic carbon content of natural ground soil was slightly higher than artificial soil. But moisture content of ground soil was lower than artificial soil. The acute toxicity test was performed for 14 days to determine the LC50 of lead and nickel in both natural garden soil and artificial soil and the LC50 value were given in table 2. In this experiment nickel shows more toxic than lead in both soil experiment. On the other hand, both metals revealed more toxic in ground soil than artificial soil.

The acid phosphatase, alkaline phosphatase and acetylcholinesterase activity of *E. fetida*

Table 1: Physicochemical parameters of the artificial soil and natural soil acts as test medium used in our experiment.

Soil parameters	Artificial soil	Natural soil
p ^H	6.40	6.80
Organic Carbon Content	0.76%	0.88%
Moisture	62.2%	61.4%

were determined after exposed to control (C), sub-lethal doses of Pb (T1 & T2), sub-lethal doses of Ni (T3 & T4), sub-lethal doses of Pb with Ni (T5 & T6). The activity of acid and alkaline phosphatases were significantly different among all sublethal doses of metallic treatments ($P < 0.05$). Activity of acid and alkaline phosphatases per mg of protein in all different treatment of soil was graphically

lowest was recorded in control set (C). Induction of acid phosphatase activity in worm was greater in nickel treated soil than the lead. The alkaline phosphatase activity was decreased in all metallic exposure than control set (C). The highest and lowest level of activity of alkaline phosphatase had been recorded in control set (C) and in T6 respectively. Inhibition of alkaline phosphatase activity in

Table 2: LC50 values of the two heavy metals (mg/kg) used in the Acute Toxicity test.

Heavy metals	Commercial compound	LC50(14 days) (mg/kg)					
		Artificial soil			Ground soil		
		LC50	95% Confidence Limit		LC50	95% Confidence Limit	
			Lower	Upper		Lower	Upper
Lead(Pb)	Lead acetate trihydrate [(CH ₃ COO) ₂ Pb. ₃ H ₂ O]	6250	1.065	1.484	6025	0.755	1.994
Nickel(Ni)	Nickel acetate Tetrahydrate [(CH ₃ .COO) ₂ Ni.4H ₂ O]	790	0.137	0.183	775	0.135	0.179

represented in figure 1 and figure 2 respectively. The highest level of acid phosphatase activity was recorded in T4 and

worm was greater in nickel treated soil than the lead. The activity of both phosphatases were more affected by combined treatment of

lead and nickel comparatively than the treatment of metal intoxication individually, lead or nickel.

4. Data analysis:

The LC50 of individual metal was determined through probit analysis by EPA probit analysis program, version 1.5 (US EPA; 2006) in 95% confidence limit of each metal. Statistical analyses for other measurements were performed by Statistical Package for Social Sciences (SPSS) version 20.0. After metallic

exposure, the mean activity of acid and alkaline phosphatases were different significantly by the analysis of variance (ANOVA) followed by Post Hoc test ($P < 0.05$). The values of both phosphatases of earthworm tissue after the metallic exposure were analyzed by correlation and showed negative type of correlation ($P < 0.01$).

5. Discussion:

Digestion and metabolism are essential part in the nutrition of animals. Both are sequential processes and maintained by several types of extra and intra cellular enzymes. Investigation of the physiological deviation from normal state made by the pollutants such as heavy metal can alter the enzymatic activity. Recent studies showed that environmental monitoring had been assessed quickly by use of enzymatic biomarkers and the increase or decrease of the activity of specific enzymes could detect a possible environment stress. The enzymatic activity acts as the excellent hallmark for potential biomonitoring out of the many biological devices to assess the metabolic alteration caused pollutants (Filimon et al., 2013). Several research had been explain alterations in the enzymatic activity were occurred (superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase etc) in several organisms under metallic stress conditions and use of

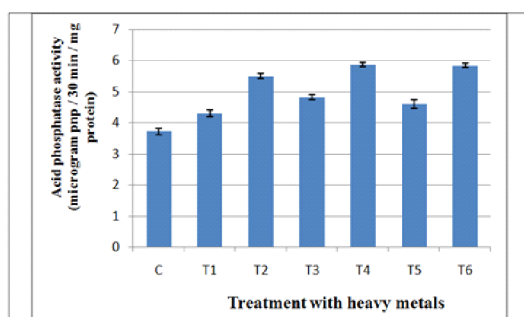


Figure 1: Acid phosphatase activity of *E. fetida* exposed to control (C), sub-lethal doses of Pb (T1 & T2), sub-lethal doses of Ni (T3 & T4), sub-lethal doses of Pb+Ni (T5 & T6). Error bar represent the standard deviation.

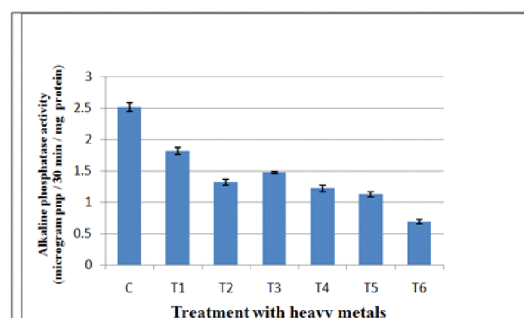


Figure 2: Alkaline phosphatase activity of *E. fetida* exposed to control (C), sub-lethal doses of Pb (T1 & T2), sub-lethal doses of Ni (T3 & T4), sub-lethal doses of Pb+Ni (T5 & T6). Error bar represent the standard deviation.

enzyme acts as efficient molecular biomarkers in evaluation of environmental impacts associated with heavy metals. (Bocchetti et al., 2008; Cogo et al., 2009). Heikens et al. (2001) pointed out a review to explain the concentration of heavy metals in most groups of soil invertebrates were happened in the order as Pb>Cd>Cu. Alnuaimi et al. (2012) carried out the activity of acid and alkaline phosphatase in the clam, *Scrobicularia plana* were altered with the treatment of heavy metals. Elevation of acid phosphatase activity indicates hyper activity of Lysosome that generally occurs in pre-necrotic changes (Novikoff 1961; De Duve 1968). Khan et al. (2014) showed that lead acetate induce the histopathological changes in brain and hepatocytes in crucian carp along with the inhibition of neural enzymes. Alam (1984) reported that the acute and chronic exposures with metals, the activity of acid phosphatase became enhanced and decrease in alkaline phosphatase activity in *Viviparus bengalensis*. Dalela et al. (1980) opined that oxidative phosphorylation became uncoupled with the inhibition of acid and alkaline phosphatases. Acid and alkaline phosphatase became decreased and increased respectively after the exposure of insecticide and herbicide (Chakravorty et al. 2015 and 2017). But Sonawane reported that (2017) after the heavy metal stress (copper, mercury and cadmium), the activity of acid and alkaline

phosphatase showed increased and decreased level respectively than control set of experiment on fresh water bivalve, *Lamellidens marginalis* and this observation support the present investigation. The both enzymes, acid and alkaline phosphatases were showed a reverse image on their specific activity after the exposure of insecticide or herbicide and heavy metals (lead and nickel). In this experimental findings, it was clearly indicated that LC50 value of lead was higher than nickel, that means nickel was more toxic than lead. After the metallic exposure, it has been also established a negative correlation between the specific activity of both acid and alkaline phosphatases of earthworm's tissue. The activity of both acid and alkaline phosphatases of earthworm's tissue were increased and decreased respectively after treatment the soil with heavy metals and acts as biochemical and physiological biomarker to identify soil pollution. The effect of nickel was more effective than lead on both phosphatases. The combination of heavy metals had synergistic effect to the activity of both phosphatases than individual metal also.

Conflict of interest: The authors declare that there are no conflicts of interest in the above specified investigation.

Acknowledgement:

Atanu Dey and Partha Pratim Chakravorty thankfully acknowledge the Principal of Raja

N. L. Khan Women's College, Midnapore, West Bengal, India for providing us with the necessary laboratory facilities and other support those are associated with this regards. Atanu Dey acknowledge the *University Scientific Instrumentation Centre (USIC)* in Vidyasagar University, Midnapore, West Bengal, India for laboratory instrumental support.

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