



METHODS OF STERILIZATION

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ABSTRACT

Sterilization (or sterilisation) refers to any process that eliminates, removes, kills, or deactivates all forms of life and other biological agents (such as fungi, bacteria, viruses, spore forms, prions, unicellular eukaryotic organisms such as Plasmodium, etc.) present in a specified region, such as a surface, a volume of fluid, medication, or in a compound such as biological culture media. A widely used method for heat sterilization is the autoclave, sometimes called a converter or steam sterilizer. Dry heat was the first method of sterilization and is a longer process than moist heat sterilization. Ethylene oxide (EO, EtO) gas treatment is one of the common methods used to sterilize, pasteurize, or disinfect items because of its wide range of material compatibility. Sterilization can be achieved using electromagnetic radiation such as electron beams, X-rays, gamma rays, or irradiation by subatomic particles. Fluids that would be damaged by heat, irradiation or chemical sterilization, such as drug products, can be sterilized by microfiltration using membrane filters.

KEYWORDS: fungi, bacteria, viruses, spore forms, prions.

INTRODUCTION

Sterilization (or sterilisation) refers to any process that eliminates, removes, kills, or deactivates all forms of life and other biological agents (such as fungi, bacteria, viruses, spore forms, prions, unicellular eukaryotic organisms such as Plasmodium, etc.) present in a specified region, such as a surface, a volume of fluid, medication, or in a compound such as biological culture media.^{[1][2]} Sterilization can be achieved through various means, including: heat, chemicals, irradiation, high pressure, and filtration. Sterilization is distinct from disinfection, sanitization, and pasteurization, in that sterilization kills, deactivates, or eliminates all forms of life and other biological agents which are present.^[3,9]

Methods of Sterilization

1. Heat Sterilization

• Steam Sterilization

A widely used method for heat sterilization is the autoclave, sometimes called a converter or steam sterilizer. Autoclaves use steam heated to 121-134 °C under pressure. To achieve sterility, the article is placed in a chamber and heated by injected steam until the article reaches a time and temperature setpoint. Almost all the air is removed from the chamber, because air is undesired in the moist heat sterilization process (this is

one trait that differ from a typical pressure cooker used for food cooking). The article is held at the temperature setpoint for a period of time which varies depending on what bioburden is present on the article being sterilized and its resistance (D-value) to steam sterilization. A general cycle would be anywhere between 3 and 15 minutes, (depending on the generated heat)^[10] at 121 °C at 100 kPa, which is sufficient to provide a sterility assurance level of 10^{-4} for a product with a bioburden of 10^6 and a D-value of 2.0 minutes.^[11] Following sterilization, liquids in a pressurized autoclave must be cooled slowly to avoid boiling over when the pressure is released. This may be achieved by gradually depressurizing the sterilization chamber and allowing liquids to evaporate under a negative pressure, while cooling the contents. Proper autoclave treatment will inactivate all resistant bacterial spores in addition to fungi, bacteria, and viruses, but is not expected to eliminate all prions, which vary in their resistance. For prion elimination, various recommendations state 121-132 °C for 60 minutes or 134 °C for at least 18 minutes. The 263K scrapie prion is inactivated relatively quickly by such sterilization procedures; however, other strains of scrapie, and strains of CJD and BSE are more resistant. Using mice as test animals, one experiment showed that heating BSE positive brain tissue at 134-138 °C for 18 minutes resulted in only a 2.5 log decrease

in prion infectivity.^[12] Most autoclaves have meters and charts that record or display information, particularly temperature and pressure as a function of time. The information is checked to ensure that the conditions required for sterilization have been met. Indicator tape is often placed on packages of products prior to autoclaving, and some packaging incorporates indicators. The indicator changes color when exposed to steam, providing a visual confirmation. Bioindicators can also be used to independently confirm autoclave performance. Simple bioindicator devices are commercially available based on microbial spores. Most contain spores of the heat resistant microbe *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*), which is extremely resistant to steam sterilization. Biological indicators may take the form of glass vials of spores and liquid media, or as spores on strips of paper inside glassine envelopes. These indicators are placed in locations where it is difficult for steam to reach to verify that steam is penetrating there. For autoclaving, cleaning is critical. Extraneous biological matter or grime may shield organisms from steam penetration. Proper cleaning can be achieved through physical scrubbing, sonication, ultrasound, or pulsed air.^[13] Pressure cooking and canning is analogous to autoclaving, and when performed correctly renders food sterile. Moist heat causes the destruction of microorganisms by denaturation of macromolecules, primarily proteins. This method is a faster process than dry heat sterilization.

• Dry Heat Sterilization

Dry heat was the first method of sterilization and is a longer process than moist heat sterilization. The destruction of microorganisms through the use of dry heat is a gradual phenomenon. With longer exposure to lethal temperatures, the number of killed microorganisms increases. Forced ventilation of hot air can be used to increase the rate at which heat is transferred to an organism and reduce the temperature and amount of time needed to achieve sterility. At higher temperatures, shorter exposure times are required to kill organisms. This can reduce heat-induced damage to food products.^[14] The standard setting for a hot air oven is at least two hours at 160 °C. A rapid method heats air to 190 °C for 6 minutes for unwrapped objects and 12 minutes for wrapped objects.^{[15][16]} Dry heat has the advantage that it can be used on powders and other heat-stable items that are adversely affected by steam (e.g. it does not cause rusting of steel objects).

• Flaming Sterilization

Flaming is done to loops and straight-wires in microbiology labs. Leaving the loop in the flame of a Bunsen burner or alcohol lamp until it glows red ensures that any infectious agent is inactivated. This is commonly used for small metal or glass objects, but not for large objects (see Incineration below). However, during the initial heating infectious material may be sprayed from the wire surface before it is killed,

contaminating nearby surfaces and objects. Therefore, special heaters have been developed that surround the inoculating loop with a heated cage, ensuring that such sprayed material does not further contaminate the area. Another problem is that gas flames may leave carbon or other residues on the object if the object is not heated enough. A variation on flaming is to dip the object in 70% or higher ethanol, then briefly touch the object to a Bunsen burner flame. The ethanol will ignite and burn off rapidly, leaving less residue than a gas flame.

• Incineration

Incineration is a waste treatment process that involves the combustion of organic substances contained in waste materials. This method also burns any organism to ash. It is used to sterilize medical and other biohazardous waste before it is discarded with non-hazardous waste. Bacteria incinerators are mini furnaces that incinerate and kill off any microorganisms that may be on an inoculating loop or wire.^[17]

• Tyndallization

Named after John Tyndall, Tyndallization^[18] is an obsolete and lengthy process designed to reduce the level of activity of sporulating bacteria that are left by a simple boiling water method. The process involves boiling for a period (typically 20 minutes) at atmospheric pressure, cooling, incubating for a day, and then repeating the process a total of three to four times. The incubation periods are to allow heat-resistant spores surviving the previous boiling period to germinate to form the heat-sensitive vegetative (growing) stage, which can be killed by the next boiling step. This is effective because many spores are stimulated to grow by the heat shock. The procedure only works for media that can support bacterial growth and will not sterilize non-nutritive substrates like water. Tyndallization is also ineffective against prions.

• Glass Bead Sterilizers

Glass bead sterilizers work by heating glass beads to 250 °C. Instruments are then quickly doused in these glass beads, which heat the object while physically scraping contaminants off their surface. Glass bead sterilizers were once a common sterilization method employed in dental offices as well as biological laboratories,^[19] but are not approved by the U.S. Food and Drug Administration (FDA) and Centers for Disease Control and Prevention (CDC) to be used as a sterilizers since 1997.^[20] They are still popular in European and Israeli dental practices although there are no current evidence-based guidelines for using this sterilizer.^[19]

2. Chemical Sterilization

Chemicals are also used for sterilization. Heating provides a reliable way to rid objects of all transmissible agents, but it is not always appropriate if it will damage heat-sensitive materials such as biological materials, fibre optics, electronics, and many plastics. In

these situations, chemicals, either as gases or in liquid form, can be used as sterilant. While the use of gas and liquid chemical sterilant avoids the problem of heat damage, users must ensure that article to be sterilized is chemically compatible with the sterilant being used. In addition, the use of chemical sterilant poses new challenges for workplace safety, as the properties that make chemicals effective sterilant usually make them harmful to humans.

• Ethylene oxide

Ethylene oxide (EO, EtO) gas treatment is one of the common methods used to sterilize, pasteurize, or disinfect items because of its wide range of material compatibility. It is also used to process items that are sensitive to processing with other methods, such as radiation (gamma, electron beam, X-ray), heat (moist or dry), or other chemicals. Ethylene oxide treatment is the most common sterilization method, used for approximately 70% of total sterilizations, and for over 50% of all disposable medical devices.^[21] Ethylene oxide treatment is generally carried out between 30 °C and 60 °C with relative humidity above 30% and a gas concentration between 200 and 800 mg/l.^[22] Typically, the process lasts for several hours. Ethylene oxide is highly effective, as it penetrates all porous materials, and it can penetrate through some plastic materials and films. Ethylene oxide kills all known microorganisms such as bacteria (including spores), viruses, and fungi (including yeasts and moulds), and is compatible with almost all materials even when repeatedly applied. It is flammable, toxic, and carcinogenic; however, with a reported potential for some adverse health effects when not used in compliance with published requirements. Ethylene oxide sterilizers and processes require biological validation after sterilizer installation, significant repairs or process changes. The traditional process consists of a preconditioning phase (in a separate room or cell), a processing phase (more commonly in a vacuum vessel and sometimes in a pressure rated vessel), and an aeration phase (in a separate room or cell) to remove ethylene oxide residues and lower by-products such as ethylene chlorohydrin (EC or ECH) and, of lesser importance, ethylene glycol (EG). An alternative process, known as all-in-one processing, also exists for some products whereby all three phases are performed in the vacuum or pressure rated vessel. This latter option can facilitate faster overall processing time and residue dissipation. The most common ethylene oxide processing method is the gas chamber method. To benefit from economies of scale, ethylene oxide has traditionally been delivered by filling a large chamber with a combination of gaseous ethylene oxide either as pure ethylene oxide, or with other gases used as diluents (chlorofluorocarbons (CFCs), hydrochlorofluorocarbons (HCFCs), or carbon dioxide). Ethylene oxide is still widely used by medical device manufacturers. Since ethylene oxide is explosive at concentrations above 3%,^[23] ethylene oxide was traditionally supplied with an inert carrier gas such as a CFC or HCFC. The use of

CFCs or HCFCs as the carrier gas was banned because of concerns of ozone depletion.^[24] These halogenated hydrocarbons are being replaced by systems using 100% ethylene oxide because of regulations and the high cost of the blends. In hospitals, most ethylene oxide sterilizers use single use cartridges because of the convenience and ease of use compared to the former plumbed gas cylinders of ethylene oxide blends. It is important to adhere to patient and healthcare personnel government specified limits of ethylene oxide residues in and/or on processed products, operator exposure after processing, during storage and handling of ethylene oxide gas cylinders, and environmental emissions produced when using ethylene oxide. The U.S. Occupational Safety and Health Administration (OSHA) has set the permissible exposure limit (PEL) at 1 ppm calculated as an eight-hour time weighted average (TWA) [29 CFR 1910.1047] and 5 ppm as a 15-minute excursion limit (EL). The National Institute for Occupational Safety and Health (NIOSH) immediately dangerous to life and health limit (IDLH) for ethylene oxide is 800 ppm.^[25] The odor threshold is around 500 ppm,^[26] so ethylene oxide is imperceptible until concentrations well above the OSHA PEL. Therefore, OSHA recommends that continuous gas monitoring systems be used to protect workers using ethylene oxide for processing.^[27] Employees' health records must be maintained during employment and after termination of employment for 30 years.

• Nitrogen Dioxide

Nitrogen dioxide (NO₂) gas is a rapid and effective sterilant for use against a wide range of microorganisms, including common bacteria, viruses, and spores. The unique physical properties of NO₂ gas allow for sterilant dispersion in an enclosed environment at room temperature and ambient pressure. The mechanism for lethality is the degradation of DNA in the spore core through nitration of the phosphate backbone, which kills the exposed organism as it absorbs NO₂. This degradation occurs at even very low concentrations of the gas.^[28] NO₂ has a boiling point of 21 °C at sea level, which results in a relatively high saturated vapour pressure at ambient temperature. Because of this, liquid NO₂ may be used as a convenient source for the sterilant gas. Liquid NO₂ is often referred to by the name of its dimer, dinitrogen tetroxide (N₂O₄). Additionally, the low levels of concentration required, coupled with the high vapour pressure, assures that no condensation occurs on the devices being sterilized. This means that no aeration of the devices is required immediately following the sterilization cycle.^[29] NO₂ is also less corrosive than other sterilant gases, and is compatible with most medical materials and adhesives.^[29] The most-resistant organism (MRO) to sterilization with NO₂ gas is the spore of *Geobacillus stearothermophilus*, which is the same MRO for both steam and hydrogen peroxide sterilization processes. The spore form of *G. stearothermophilus* has been well characterized over the years as a biological indicator in sterilization applications. Microbial inactivation of *G.*

stearothermophilus with NO₂ gas proceeds rapidly in a log-linear fashion, as is typical of other sterilization processes. Noxilizer, Inc. has commercialized this technology to offer contract sterilization services for medical devices at its Baltimore, Maryland (U.S.) facility.^[30] This has been demonstrated in Noxilizer's lab in multiple studies and is supported by published reports from other labs. These same properties also allow for quicker removal of the sterilant and residuals through aeration of the enclosed environment. The combination of rapid lethality and easy removal of the gas allows for shorter overall cycle times during the sterilization (or decontamination) process and a lower level of sterilant residuals than are found with other sterilization methods.^[29]

• Ozone

Ozone is used in industrial settings to sterilize water and air, as well as a disinfectant for surfaces. It has the benefit of being able to oxidize most organic matter. On the other hand, it is a toxic and unstable gas that must be produced on-site, so it is not practical to use in many settings. Ozone offers many advantages as a sterilant gas; ozone is a very efficient sterilant because of its strong oxidizing properties (E= 2.076 vs SHE^[31]) capable of destroying a wide range of pathogens, including prions without the need for handling hazardous chemicals since the ozone is generated within the sterilizer from medical grade oxygen. The high reactivity of ozone means that waste ozone can be destroyed by passing over a simple catalyst that reverts it to oxygen and ensures that the cycle time is relatively short. The disadvantage of using ozone is that the gas is very reactive and very hazardous. The NIOSH immediately dangerous to life and health limit for ozone is 5 ppm, 160 times smaller than the 800 ppm IDLH for ethylene oxide. Documentation for Immediately Dangerous to Life or Health Concentrations (IDLH): NIOSH Chemical Listing and Documentation of Revised IDLH Values (as of 3/1/95)^[32] and OSHA has set the PEL for ozone at 0.1 ppm calculated as an 8 hour time weighted average (29 CFR 1910.1000, Table Z-1). The Canadian Center for Occupation Health and Safety provides an excellent summary of the health effects of exposure to ozone. The sterilant gas manufacturers include many safety features in their products but prudent practice is to provide continuous monitoring to below the OSHA PEL to provide a rapid warning in the event of a leak. Monitors for determining workplace exposure to ozone are commercially available.

• Glutaraldehyde and formaldehyde

Glutaraldehyde and formaldehyde solutions (also used as fixatives) are accepted liquid sterilizing agents, provided that the immersion time is sufficiently long. To kill all spores in a clear liquid can take up to 22 hours with glutaraldehyde and even longer with formaldehyde. The presence of solid particles may lengthen the required period or render the treatment ineffective. Sterilization of blocks of tissue can take much longer, due to the time required for the fixative to penetrate. Glutaraldehyde and

formaldehyde are volatile, and toxic by both skin contact and inhalation. Glutaraldehyde has a short shelf life (<2 weeks) and is expensive. Formaldehyde is less expensive and has a much longer shelf life if some methanol is added to inhibit polymerization to paraformaldehyde but is much more volatile. Formaldehyde is also used as a gaseous sterilizing agent; in this case, it is prepared on-site by depolymerization of solid paraformaldehyde. Many vaccines, such as the original Salk polio vaccine, are sterilized with formaldehyde.

• Hydrogen Peroxide

Hydrogen peroxide, in both liquid and as vaporized hydrogen peroxide (VHP), is another chemical sterilizing agent. Hydrogen peroxide is strong oxidant, which allows it to destroy a wide range of pathogens. Hydrogen peroxide is used to sterilize heat or temperature sensitive articles such as rigid endoscopes. In medical sterilization hydrogen peroxide is used at higher concentrations, ranging from around 35% up to 90%. The biggest advantage of hydrogen peroxide as a sterilant is the short cycle time. Whereas the cycle time for ethylene oxide may be 10 to 15 hours, some modern hydrogen peroxide sterilizers have a cycle time as short as 28 minutes.^[33] Drawbacks of hydrogen peroxide include material compatibility, a lower capability for penetration and operator health risks. Products containing cellulose, such as paper, cannot be sterilized using VHP and products containing nylon may become brittle.^[34] The penetrating ability of hydrogen peroxide is not as good as ethylene oxide and so there are limitations on the length and diameter of lumens that can be effectively sterilized and guidance is available from the sterilizer manufacturers. Hydrogen peroxide is primary irritant and the contact of the liquid solution with skin will cause bleaching or ulceration depending on the concentration and contact time. It is relatively non-toxic when diluted to low concentrations but is a dangerous oxidizer at high concentrations (> 10% w/w). The vapour is also hazardous, primarily affecting the eyes and respiratory system. Even short term exposures can be hazardous and NIOSH has set the Immediately Dangerous to Life and Health Level (IDLH) at 75 ppm,^[25] less than one tenth the IDLH for ethylene oxide (800 ppm). Prolonged exposure to lower concentrations can cause permanent lung damage and consequently, OSHA has set the permissible exposure limit to 1.0 ppm, calculated as an 8-hour time weighted average.^[35] Sterilizer manufacturers go to great lengths to make their products safe through careful design and incorporation of many safety features, though there are still workplace exposures of hydrogen peroxide from gas sterilizers are documented in the FDA MAUDE database.^[36] When using any type of gas sterilizer, prudent work practices will include good ventilation, a continuous gas monitor for hydrogen peroxide and good work practices and training.^{[37][38]} Vaporized hydrogen peroxide (VHP) is used to sterilize large enclosed and sealed areas such as entire rooms and aircraft interiors. Although toxic, VHP

breaks down in a short time from H_2O_2 to water $\text{H}_2\text{O} + \text{O}_2$.

- **Peracetic Acid**

Peracetic acid (0.2%) is a recognized sterilant by the FDA^[39] for use in sterilizing medical devices such as endoscopes.

Potential for chemical sterilization of prions

Prions are highly resistant to chemical sterilization. Treatment with aldehydes such as formaldehyde have actually been shown to increase prion resistance. Hydrogen peroxide (3%) for one hour was shown to be ineffective, providing less than 3 logs (10^{-3}) reduction in contamination. Iodine, formaldehyde, glutaraldehyde, and peracetic acid also fail this test (one hour treatment). Only chlorine, phenolic compounds, guanidinium thiocyanate, and sodium hydroxide (NaOH) reduce prion levels by more than 4 logs; chlorine (too corrosive to use on certain objects) and NaOH are the most consistent. Many studies have shown the effectiveness of sodium hydroxide.^[40]

3. Radiation Sterilization

Sterilization can be achieved using electromagnetic radiation such as electron beams, X-rays, gamma rays, or irradiation by subatomic particles.^[41] Electromagnetic or particulate radiation can be energetic enough to ionize atoms or molecules (ionizing radiation), or less energetic (non-ionizing radiation).

- **Non-ionizing Radiation Sterilization**

Ultraviolet light irradiation (UV, from a germicidal lamp) is useful for sterilization of surfaces and some transparent objects. Many objects that are transparent to visible light absorb UV. UV irradiation is routinely used to sterilize the interiors of biological safety cabinets between uses, but is ineffective in shaded areas, including areas under dirt (which may become polymerized after prolonged irradiation, so that it is very difficult to remove). It also damages some plastics, such as polystyrene foam if exposed for prolonged periods of time.

- **Ionizing Radiation Sterilization**

The safety of irradiation facilities is regulated by the United Nations International Atomic Energy Agency and monitored by the different national Nuclear Regulatory Commissions. The incidents that have occurred in the past are documented by the agency and thoroughly analyzed to determine root cause and improvement potential. Such improvements are then mandated to retrofit existing facilities and future design. Gamma radiation is very penetrating, and is commonly used for sterilization of disposable medical equipment, such as syringes, needles, cannulas and IV sets, and food. It is emitted by a radioisotope, usually cobalt-60 (^{60}Co) or caesium-137 (^{137}Cs), which have photon energies of up to 1.3 and 0.66 MeV respectively. Use of a radioisotope requires shielding for the safety of the

operators while in use and in storage. With most designs, the radioisotope is lowered into a water-filled source storage pool, which absorbs radiation and allows maintenance personnel to enter the radiation shield. One variant keeps the radioisotope under water at all times and lowers the product to be irradiated into the water towards the source in hermetic bells; no further shielding is required for such designs. Other uncommonly used designs use dry storage, providing movable shields that reduce radiation levels in areas of the irradiation chamber. An incident in Decatur Georgia, US, where water-soluble caesium-137 leaked into the source storage pool, requiring NRC intervention^[42] has led to use of this radioisotope being almost entirely discontinued in favour of the more costly, non-water-soluble cobalt-60. Cobalt-60 gamma photons have about twice the energy, and hence greater penetrating range, of Caesium-137 radiation. Electron beam processing is also commonly used for sterilization. Electron beams use an on-off technology and provide a much higher dosing rate than gamma or x-rays. Due to the higher dose rate, less exposure time is needed and thereby any potential degradation to polymers is reduced. Because electrons carry a charge, electron beams are less penetrating than either gamma or x-rays. Facilities rely on substantial concrete shields to protect workers and the environment from radiation exposure. X-rays: high-energy X-rays (produced by bremsstrahlung) allow irradiation of large packages and pallet loads of medical devices. They are sufficiently penetrating to treat multiple pallet loads of low-density packages with very good dose uniformity ratios. X-ray sterilization does not require chemical or radioactive material: high-energy X-rays are generated at high intensity by an X-ray generator that does not require shielding when not in use. X-rays are generated by bombarding a dense material (target) such as tantalum or tungsten with high-energy electrons in a process known as bremsstrahlung conversion. These systems are energy-inefficient, requiring much more electrical energy than other systems for the same result. Irradiation with X-rays, gamma rays, or electrons does not make materials radioactive, because the energy used is too low. Generally an energy of at least 10 MeV is needed to induce radioactivity in a material.^[43] Neutrons and very high-energy particles can make materials radioactive, but have good penetration, whereas lower energy particles (other than neutrons) cannot make materials radioactive, but have poorer penetration. Sterilization by irradiation with gamma rays may however in some cases affect material properties.^[44] Irradiation is used by the United States Postal Service to sterilize mail in the Washington, D.C. area. Some foods (e.g. spices, ground meats) are sterilized by irradiation.^[45] Subatomic particles may be more or less penetrating and may be generated by a radioisotope or a device, depending upon the type of particle.

4. Sterile Filtration

Fluids that would be damaged by heat, irradiation or chemical sterilization, such as drug products, can be

sterilized by microfiltration using membrane filters. This method is commonly used for heat labile pharmaceuticals and protein solutions in medicinal drug processing. A microfilter with pore size 0.2 μm will usually effectively remove microorganisms.^[46] Some staphylococcal species have, however, been shown to be flexible enough to pass through 0.22 μm filters.^[47] In the processing of biologics, viruses must be removed or inactivated, requiring the use of nanofilters with a smaller pore size (20-50 nm) are used. Smaller pore sizes lower the flow rate, so in order to achieve higher total throughput or to avoid premature blockage, pre-filters might be used to protect small pore membrane filters. Tangential flow filtration (TFF) and alternating tangential flow (ATF) systems also reduce particulate accumulation and blockage. Membrane filters used in production processes are commonly made from materials such as mixed cellulose ester or polyethersulfone (PES). The filtration equipment and the filters themselves may be purchased as pre-sterilized disposable units in sealed packaging or must be sterilized by the user, generally by autoclaving at a temperature that does not damage the fragile filter membranes. To ensure proper functioning of the filter, the membrane filters are integrity tested post-use and sometimes before use. The nondestructive integrity test assures the filter is undamaged and is a regulatory requirement.^[48] Typically, terminal pharmaceutical sterile filtration is performed inside of a cleanroom to prevent contamination.

Preservation of Sterility

Instruments that have undergone sterilization can be maintained in such condition by containment in sealed packaging until use. Aseptic technique is the act of maintaining sterility during procedures.

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