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EFFECT OF TIME AND TEMPERATURE ON NISIN ENCAPSULATED IN 50/50 NYLON/COTTON BLEND FABRIC

by
Shalli A. Sherman
and
Shaun F. Filocamo

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U.S. Army Natick Soldier Research, Development and Engineering Center
Natick, Massachusetts 01760-5000

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14. ABSTRACT

Antimicrobial textiles are becoming of increasing interest, particularly to the military, to prevent bacteria from colonizing and degrading the fabric, as well as providing protection to Soldiers from potential pathogens. Due to increasing antimicrobial resistance, alternatives to broad spectrum agents are needed. This paper discusses a novel approach of imparting antimicrobial functionality to a textile using nisin, a bacteriocin used in the food preservation industry, through encapsulation within a titania matrix. A month long storage study at conditions of 4 ºC, 25 ºC, and 37 ºC was conducted to assess the stability of the nisin on the textile over time at different temperatures. Activity of the nisin was measured weekly using a modified version of the AATCC100 method. Results showed that nisin is not active at temperatures above 4 ºC after 2 weeks and that additional or alternative encapsulation would be necessary to enhance stability of the bacteriocin.

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Preface

This report documents work by the Biological Sciences and Technology Team (BSTT) of the Warfighter Directorate at the Natick Soldier Research, Development and Engineering Center (NSRDEC) during the period from January 1, 2014 to December 31, 2015. This work involved initial investigations of nisin encapsulated in titania on a 50:50 Nylon:cotton blend (NyCo) textile. Storage and stability studies on the encapsulated materials are reported and discussed.

EFFECT OF TIME AND TEMPERATURE ON NISIN ENCAPSULATED IN 50/50 NYLON COTTON BLEND FABRIC

1 INTRODUCTION

The Biological Sciences and Technology Team (BSTT), Warfighter Directorate, of the Natick Soldier Research, Development and Engineering Center (NSRDEC) investigated the use of a naturally occurring antimicrobial, nisin, as a treatment on nylon/cotton blends to protect the Warfighter from pathogenic bacteria. This effort was part of a larger investigation, known as Functional Oxide Reactive Coatings for Enhanced Protection on Textiles (FORCE-ProTex) into creating a multifunctional textile, and work was performed from January 1, 2014 - December 31, 2015. The objective of this report is to describe the procedure used to produce the treated textile, the characterization and activity profile of these treatments over time, and the limitations encountered when using natural products.

Soldiers face threats on a day-to-day basis distinct from those that the civilian population faces. One growing concern in the military is the threat of biological pathogens. Biological threats exist in the civilian world as well, but for the Soldier especially, it would be extremely beneficial to have antimicrobial protection built into a uniform to provide an enhanced barrier against pathogens. It would also help keep the uniform cleaner and prevent biofouling, as bacteria can readily colonize fabric [1, 2]. These features are especially relevant to the Soldier, as they may have to go days or weeks without laundering.

Most of the current textile antimicrobial treatments are synthetic and nonselective, meaning they kill off potentially harmful bacteria as well as the body's own beneficial microbiota [2]. Certain textile treatments may do more harm than good, as they contribute to the growing problem of antimicrobial resistance [3]. As this resistance increases, it is necessary to investigate nontraditional approaches in order to kill bacteria selectively. One answer to this complex problem may actually come from bacteria themselves: bacteriocins.

Bacteriocins are small antimicrobial peptides produced by bacteria for use against similar strains of competing bacteria [4]. Currently, only bacteriocins found in dairy products (therefore considered "generally regarded as safe") have been used on an industrial scale for preservation in the food industry [5-7]. The most widely used bacteriocin in the food industry is nisin, a small lanthionine-containing antibiotic, or lantibiotic, containing 34 amino acids, including the unusual residues dehydrobutyrine (Dhb) and dehydroalanine (Dha) [8, 9]. The structure has been elucidated and is shown in Figure 1.

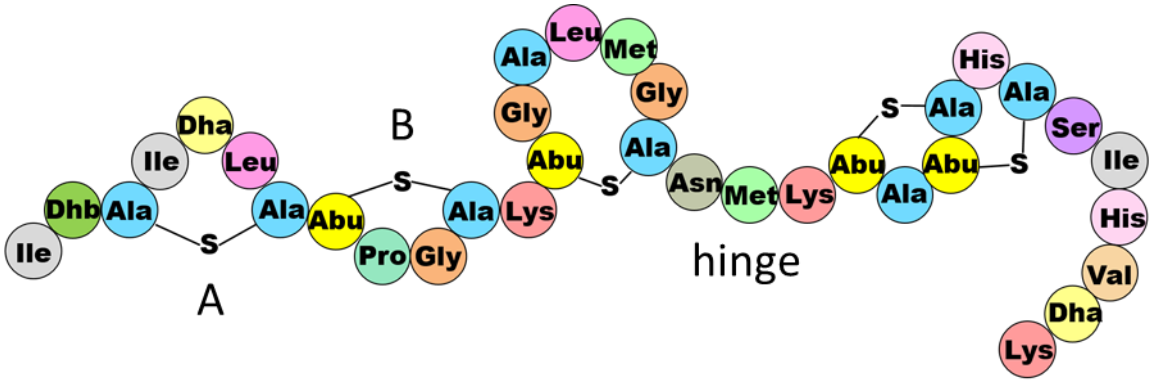

Figure 1. Structure of nisin. The A, B, and hinge regions are identified for clarity.

This structure of nisin reveals five thioether rings and minimal secondary structure. The mechanism of action for nisin involves first binding to the N-terminus to the lipid II complex, forming a pyrophosphate cage using rings A and B, and inhibiting cell wall synthesis [8, 10]. The C-terminal is then responsible for pore-formation [10]. A three amino acid hinge region exists between the N- and C-terminal domains and allows conformational changes to occur upon contacting a microbe [8].

Although several studies have investigated the structure and mechanism of action of nisin in order to better understand its stability and limitations, they have focused on nisin stability in solution, which may not directly translate to stability for other applications [8-11]. In this study, a gentle precipitation procedure has been used to entrap nisin within a titania matrix on fabric from a crude mixture containing the peptide. This titania matrix serves as a vehicle for entrapping the bacteriocin without providing any further antimicrobial activity. However, biologics in their non-native environment may be susceptible to degradation and stability issues [9]. In order to identify its limitations when used as an antimicrobial on a textile, the stability of nisin was investigated when encapsulated on a fabric swatch over time under different temperature storage conditions.

2 MATERIALS

Nisin Z was purchased from ChiHonBio at 20% purity and used without further purification. All fabric testing was done on Nyco, a 50/50 fabric blend of cotton and nylon currently used for military uniforms. Borax, sodium hydroxide, sodium phosphate dibasic, sodium phosphate monobasic, polystyrene sulfonate (PSS), polyethylenimine (PEI), and 3-chloro-2-hydroxypropyl trimethyl ammonium chloride (CHPTAC) were purchased from Sigma-Aldrich. The layer-by-layer process was completed using a KSV-NIMA automated dipcoater by Biolin Scientific. Trizma base and titanium(IV) (bisammonium lactato) dihydroxide (TBALD- 50 wt% solution in water), and nutrient broth were also purchased from Sigma Aldrich. Mannitol Salt Broth (Himedia) and bacteriological agar (Acros) were used to prepare mannitol salt agar plates for soft agar overlay studies. Dey-Engley (DE) broth was obtained from BD Falcon. *Staphyloccocus aureus* strain 27217 was obtained from American Type Culture Collection (ATCC). Biological decontamination tests were performed using a TEMPO system from Biomerieux, and TEMPO supplies were purchased from Biomerieux as well. Deionized (DI) water was obtained using a Thermo Scientific Barnstead Smart2Pure water purification system. Swatch precipitations were imaged by a Zeiss EVO 60 Scanning Electron Microscope with a tungsten filament. Energy dispersive spectroscopy (EDS) data were gathered using EDAX Genesis software to analyze the x-ray peaks generated by the sample.

3 METHODS

3.1 Preparation of Antimicrobial Swatches

3.1.1 Nyco Fabric Preparation

Nyco fabric swatches were scoured in an aqueous 2% w/v Borax solution and heated to 80 °C for 1 h to remove any residual chemicals from the manufacturing process. The swatches were placed in deionized water at the same temperature for 5 min. The swatches were then placed in a solution made up of 30 g sodium hydroxide, 150 mL DI water, and 150 mL CHPTAC. The fabric was allowed to react for 15 min, then rinsed in DI water for 15 min to provide the base positive charge. The fabric was then laid out to dry overnight. Next, the swatches underwent a layer-by-layer process, where they were dipped in solutions of deionized water, 0.1% PSS, deionized water, and 0.1% PEI (one complete layer) by the KSV NIMA dip coater to build layers of positive and negative charges. The swatches were held in each solution for 2 min, and then allowed to drip for 30 s out of solution. This process was repeated for a total of 15 layers. Swatches were allowed to dry again overnight. Once dry, the swatches were cut into 1x1 inch squares using a pneumatic dye cutter.

3.1.2 Titania precipitation/nisin encapsulation

Two separate 1 month studies were conducted on precipitated layer-by-layer swatches held at the following storage conditions: fridge (4 °C), incubator (37 °C), and room temperature (25 °C). Control swatches were stored at room temperature. Triplicate swatches were tested at each time point for each condition, so 54 swatches in total were precipitated at one time. Control swatches were precipitated using 24.5 mL of 25 mM Tris HCl buffer (pH 7.5) without nisin. For nisin encapsulation, 125 mg of 20% pure nisin was added to 24.5 mL of 25 mM Tris HCl buffer (pH 7.5) to create a 1 mg/mL concentration in terms of pure nisin. This solution was vortexed for 5 min, then spun down in a centrifuge at 3,500 rpm at 4 °C for 10 min in order to pellet the insoluble proteins. The supernatant was poured off into a petri dish, and six layer-by-layered swatches were added. All dishes (control and nisin) were placed on an orbital shaker at 250 rpm, and once the swatches had been wetted, 0.5 mL of TBALD were added (1% titania). Swatches were allowed to precipitate for 6 h. The reaction solution was then poured off and replaced with 25 mL of DI water. Swatches were rinsed for 5 min. This process was repeated twice more for a total of three rinses. Three control swatches and three nisin swatches were inoculated immediately following the final rinse for time zero testing. Remaining swatches were then transferred into large sterile plastic dishes, wrapped in aluminum foil, and placed in their respective storage conditions (4 °C, 37 °C, or 25 °C). Control swatches and room temperature nisin swatches were left on the bench and their storage containers were wrapped in aluminum foil to protect from potential UV light exposure, which could negatively impact the nisin activity.

3.2 Biological Decontamination Testing

3.2.1 Modified AATCC147 (preliminary testing)

1x1 inch Nyco swatches were precipitated according to the above procedure. 20 µL of *S. aureus* (grown to an optical density of 1 at 600 nm) were added to 7 mL of mannitol salt soft agar. The

solution was gently mixed, and then poured over onto a mannitol salt agar plate. The soft agar was allowed to dry, and then a precipitated swatch was laid over and incubated overnight at 37 °C. Plates were checked for a zone of clearing around the swatch.

3.2.2 AATCC100

A modified version of the AATCC100 method was used to obtain quantitative results of the storage conditions. Two separate 1 month studies were conducted on precipitated swatches held at the aforementioned storage conditions. Time points tested were 0 days, 7 days, 14 days, 21 days, and 28 days. Immediately following precipitation of swatches, three control swatches and three nisin swatches were inoculated. For inoculation, *S. aureus* (ATCC 27217) was grown from frozen glycerol stocks in 10 mL of nutrient broth and incubated for 6 h on a 37 °C shaker. Once an optical density of 1 had been achieved (approximately 10^7 colony forming units [CFUs]), the culture was diluted by a factor of 10 into nutrient broth. 100 µL of the diluted culture was used to inoculate the precipitated swatches. Swatches were inoculated in sterile plastic petri dishes, and then placed in an incubator at 37 °C for 18 h. They were then placed into 5 mL of DE neutralizing broth and vortexed for 5 min to allow bacteria to fall off into solution. Serial dilutions up to 10^{-4} for controls and 10^{-2} for nisin samples were created using 20 mM sodium phosphate buffer (pH 7.2). Solutions were then tested using TEMPO selective *S. aureus* media according to manufacturer's instructions. The TEMPO system has been validated by the Association of Analytical Communities Research Institute as a Performance-Tested Method. TEMPO STA is an automated test for the enumeration of gram-positive staphylococci (*S. aureus*) based on the format of the TEMPO Most Probable Number (MPN) procedure (miniaturized card containing 48 wells across 3 different dilutions for the automatic determination of the MPN. The system fills the card, and after 24-27 h of incubation at 37 °C, automatically reads the card and calculates the MPN as CFU/mL. TEMPO cards were incubated at 37 °C for 24 h, then read in the TEMPO reader [12]. For every time point after time zero, all three nisin storage conditions and controls swatches were tested according to the above procedure.

4 RESULTS AND DISCUSSION

4.1 Modification and Functionalization of Textile Surface

After a 6 h precipitation of titania onto a PEI terminated swatch, SEM/EDS is used to confirm the presence of a titanium-containing precipitate (Figure 2). The amine group in the PEI, which under neutral conditions has a positive charge, provides a reactive site for titania to precipitate on and bind to the Nyco fabric. There is a significant difference in the roughness of the Nyco surface versus a scoured, unreacted control (Figure 2a and 2c). Furthermore, the presence of titanium in the coating is evidenced by an increase in the TiK peak in EDS spectra versus the control (Figure 2b and 2d). A consistent, continuous coating is necessary to prevent the PEI from leaching out from the fibers. The free amine groups in the PEI can exhibit indiscriminate antimicrobial properties, which could confuse the results of a titania coating with encapsulated nisin. Antimicrobial overlays were used to confirm the titania coating was providing adequate coverage.

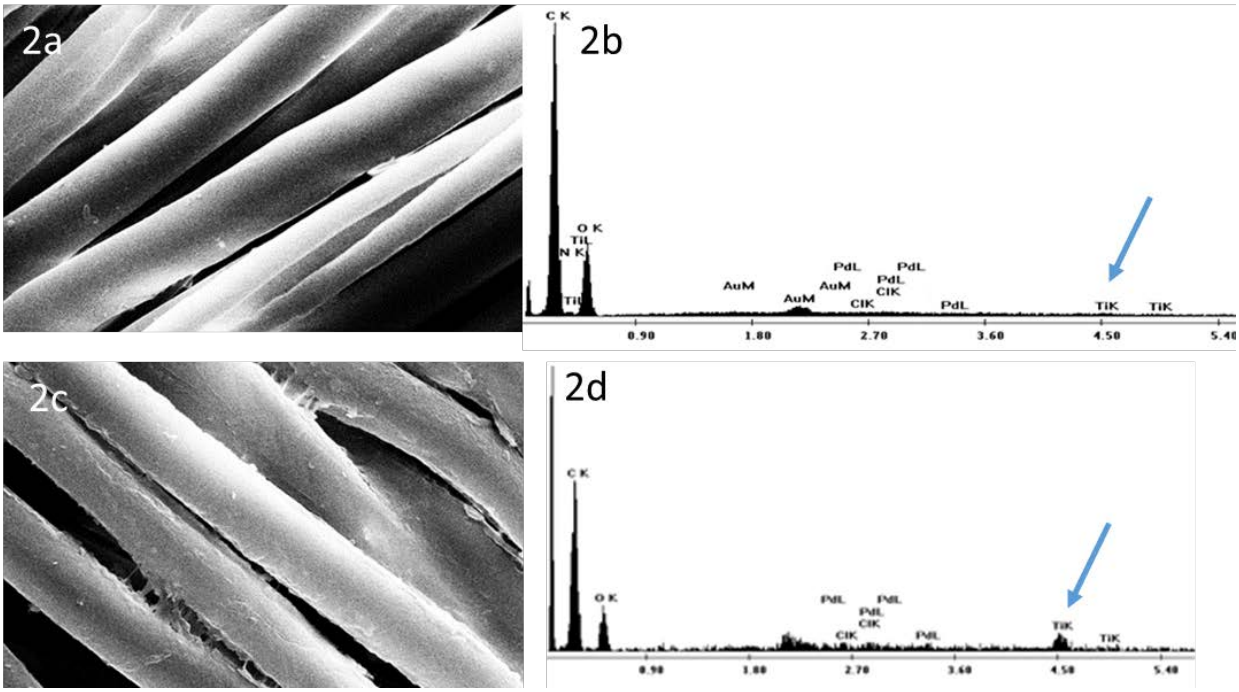

Figure 2. SEM and EDS results of scoured swatches (A and B) and PEI and titania precipitated swatches (C and D). SEM image reveals no coating on the scoured fibers (A) and titania on the coated fibers. EDS data in (D) reveals a significant titanium (TiK) peak, indicating presence of titanium in the coating. An arrow has been added to identify the location of the TiK peaks.

4.2 Antimicrobial Activity and Decontamination Testing

Overlays were done on precipitated swatches in the absence and presence of nisin, which provided a qualitative assessment of antimicrobial activity. Figure 3a, representing a swatch

containing no nisin, showed no zone of clearing around the edge, indicating that there was no leaching occurring from the PEI through the titania coating. In contrast, Figure 3b, representing a swatch precipitated in the presence of nisin, showed a halo of inhibition surrounding the swatch where the antimicrobial leached and prevented the bacteria from growing. This indicates that some nisin has been encapsulated, but is still accessible for activity.

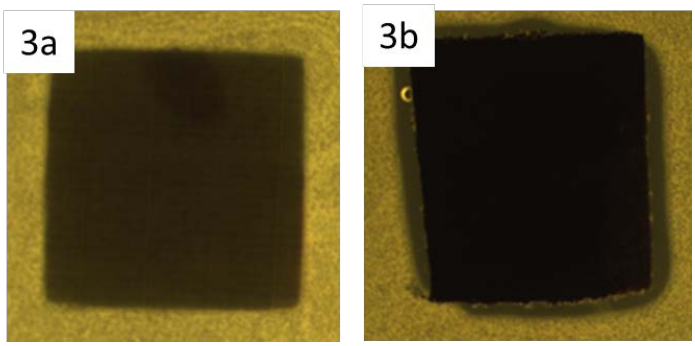

Figure 3. Image 3a depicts a 6 h precipitated titania swatch. No antimicrobial activity is observed. Image 3b depicts a zone of clearing around a 6 h precipitated titania swatch with 1 mg/mL of nisin, indicating antimicrobial activity.

Quantitative results were obtained through a modified version of the AATCC100 method. Three swatches per storage condition per time point were tested and averaged to obtain quantitative results. A swatch precipitated with titania only was used as a control rather than 0 and 24 h time points. For baseline values, swatches were tested immediately following precipitation and demonstrated a log kill of over 4. As seen in Figure 4, antimicrobial activity over time is highly dependent on storage temperature. After 7 days, activity was comparable for 25 ºC and 4 ºC conditions and appeared to actually increase from 0 days, yet at 37 ºC activity dropped by 80% from the baseline value. After 14 days, 4 ºC and 25 ºC showed little change, but 37 ºC activity had dropped 95% from the baseline value. It was not until day 21 that a significant difference in activity could be observed between the 4 ºC and 25 ºC storage conditions. After 28 days, only the 4 ºC condition demonstrated significant activity, dropping only 3% of the baseline value. The 37 ºC swatches did not show activity, and 25 ºC swatches had dropped 73% from the baseline value. For all log kill measurements, the standard deviations fell between 0.00 and 0.868, with the smallest ranges occurring within the 4 °C storage set, as seen in Table 1.

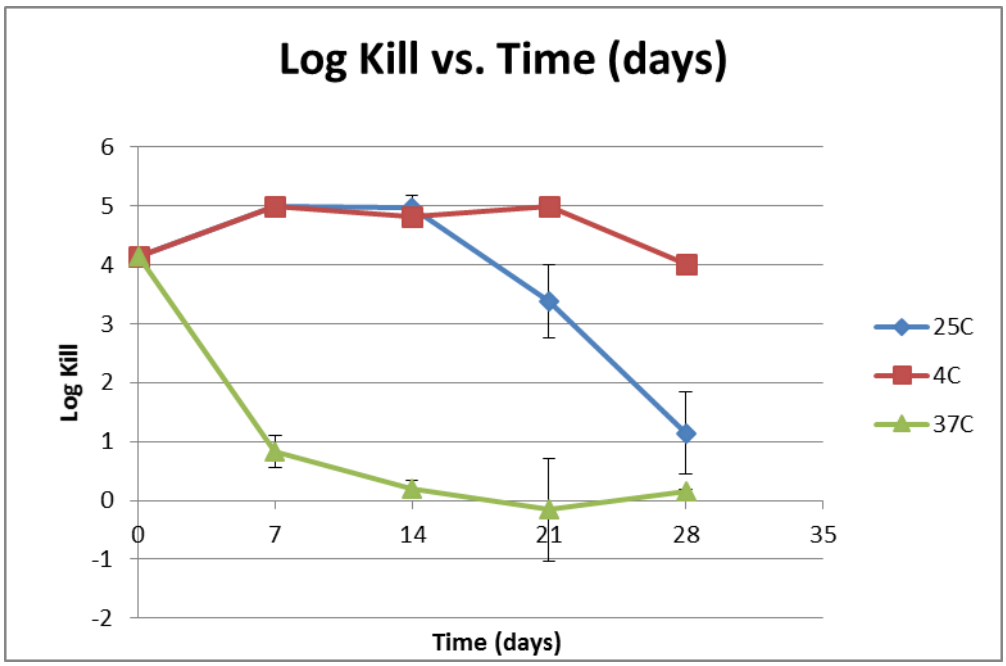

Figure 4. Log kill over time for different storage conditions. Note that some error bars are smaller than the symbol.

Table 1. Log kill and standard deviation values. Standard deviation of 0 indicates that the bacteria in the sample were too few to count.

Storage Condition	Day 0	Day 7	Day 14	Day 21	Day 28
25ºC	4.15 ± .264	5 ± 0	4.969 ± .208	3.379 ± .616	1.141 ± .703
4ºC	4.15 ± .264	5 ± 0	4.815 ± 0	5 ± 0	4.021 ± .072
37ºC	4.15 ± .264	0.825 ± .277	0.207 ± .140	-0.155 ± .868	0.155 ± .040

It is apparent from this month long study that storage temperature highly impacts the antimicrobial activity of nisin on a textile. Significant loss of activity is observed after two weeks, if not sooner, at any temperature above 4 ºC. This example of environmental instability is consistent with other results, although to the knowledge of the authors, this is the first study to look at these effects on fabric [9].

Studies on nisin degradation products have found that the Dha residue is particularly susceptible to degradation, which can lead to a loss in biological activity [9]. In another study, it was demonstrated that after reaction with nucleophiles the Dha and Dhb residues are modified, leading to a complete loss of biological activity [11]. As both Dha and Dhb residues are incorporated into the ring structures, it is possible that modification of these residues through oxidation or hydrolysis leads to a deformation of the ring and loss of antimicrobial activity according to the known mechanism. Other papers have illustrated that the ring structures are susceptible to oxidations and/or nucleophilic attack even at room temperature, which is consistent with the observed results [9]. Though nisin is susceptible to degradation and loss of activity, the described encapsulation of the peptide imparts antimicrobial functionality to a textile

in a novel way, and with adequate environmental protection, illustrates a potential path to targeted antimicrobial protection on textiles.

5 CONCLUSION

In this study, the efficacy of using antimicrobial biologics on a textile has been successfully demonstrated. The encapsulation method, which uses a metal oxide matrix, is a novel approach to impart an antimicrobial functionality. However, it is not effective in stabilizing this biologic at temperatures above 4 ºC. Therefore, for this to be a viable technology, a secondary encapsulation or co-encapsulation would likely be necessary. Notwithstanding, this encapsulation method was able to provide an antimicrobial functionality to the textile without any initial damage to the nisin. It is apparent that degradation only occurs over time from environmental factors rather than the entrapment process. Storage at lower temperatures seems to delay the degradation process, indicating that with protective encapsulation biologics could be an effective additive to textiles.

From this study, it is apparent that biologics may not behave as expected once isolated from their native environment. However, the positive results at 4 ºC storage provide insight into how to better stabilize biologics for long-term use. Nisin, as a representative for many isolated biologics, exhibits loss of activity in elevated temperatures and more basic pH environments [9]. Activity over time is dependent on storage conditions, with stronger activity correlating to lower temperatures. Future work should focus on alternative encapsulation strategies to better protect the biologic so it can retain activity at a wider range of temperatures over time.

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