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Microbial sterility testing of oil-formulated bovine somatotropin using Tween® 80 dispersion

Wesley E. Workman[a,*], Robert A. Clayton[b]

[a]*Protiva, Monsanto Company, 700 Chesterfield Parkway North, Chesterfield, MO 63198, USA*
[b]*Monsanto Corporate Research, Monsanto Company, 700 Chesterfield Parkway North, Chesterfield, MO 63198, USA*

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Abstract

The Food and Drug Administration requires that sterile pharmaceutical products be free of viable microorganisms. Sterility testing of pharmaceutical products provides added assurance that the product is sterile. Sterility testing is typically done by inoculating the drug product into microbial growth media followed by visual inspection for growth during incubation for a specified time period. A lack of visual growth indicates that the drug product samples tested were sterile. Formulated Posilac® bovine somatotropin[1] consists of protein particles suspended in an oil-based excipient. The product formulation is immiscible in aqueous media due to the excipient's water insolubility and the insolubility of the protein particles at near neutral pH values. Because the formulation is packaged and sold as a sterile product, it is critical that a sensitive microbial sterility test method be used for this key quality test. A sterility test method was developed for Posilac® that utilized Tween® 80 (i.e. polysorbate 80) as a dispersant. Dispersion of the product using Tween® 80 produced a homogeneous suspension of bovine somatotropin particles and oil droplets in the micron size range. The suspension did not appreciably settle out with time, attesting to the homogeneous nature of the mixture. This method was found to be compatible with survival, recovery, and growth from low numbers of the test organisms required by the U.S. Pharmacopeia XXIII as well as from two additional test cultures.

Keywords: Bacteria; Microorganism; Oil formulation; Pharmaceutical sterility.

1. Introduction

The formulated Posilac® bovine somatropin consists of protein particles suspended in an excipient that consists of mostly sesame oil. The product is a prolonged release formulation administered to dairy cattle by injection to increase milk production. The oil in the product renders the product immiscible in aqueous solution. This immiscibility complicates sterility testing of the product because aqueous microbiological media

* Corresponding author. Tel.: (+1)314-537-6749.
[1] Posilac® is a registered trademark of Monsanto Company.

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are used for testing. Complete solubilization of the product cannot be achieved in microbiological media because the bovine somatotropin protein particles are not soluble in an aqueous environment compatible with the growth of microorganisms (i.e. neutral pH). The United States Pharmacopeia (USP) XXIII describes techniques using filtration to remove contaminating microorganisms from oil-based pharmaceuticals for subsequent culture [1]. These filtration techniques cannot be used here because the suspended protein particles easily blocked the filter. Therefore, to conduct sterility testing it is desirable to produce a homogenous emulsion/suspension of the product in microbiological media to enhance recovery of viable microorganisms, if present, from the product.

The USP XXIII [1] discusses the sterility testing of oil-based pharmaceuticals and indicates that one should use an "...aqueous vehicle capable of dispersing the test material homogeneously throughout the fluid mixture..." for direct transfer testing. For membrane filtration sterility testing of oils, USP XXIII suggests the use of an aqueous medium containing 0.1% w/v polysorbate 80 as the final culture medium. The British Pharmacopoeia 1993 [2] suggests "...for oily liquids use media to which have been added 0.1% w/v of (4-*tert*-octylphenoxy) polyethoxyethanol or 1% w/v of polysorbate 80 or another suitable emulsifying agent, in an appropriate concentration, shown not to have any antimicrobial properties under the condition of the test...". Tween® 80 is polysorbate 80 and (4-*tert*-octylphenoxy) polyethoxy-ethanol is a Triton® surfactant. In an attempt to follow the British Pharmacopoeia 1993, 1% w/v Tween® 80 was added to the microbiological media used to suspend the product. The addition of 1% w/v Tween® 80 to the microbiological media did not produce a homogeneous emulsion upon addition of the formulated product followed by vigorous agitation.

The Bacteriological Analytical Manual [3], a Food and Drug Administration (FDA) publication, contains a chaper (i.e. Chapter 25) outlining microbiological methods for cosmetics. This chapter indicates that oil-based products can be assayed for microorganisms by mixing 1 g of the product with 1 g of Tween® 80 followed by dilution into the appropriate microbiological media. This technique was tested with the formulated bovine somatotropin product and was found to produce a homogeneous emulsion containing protein particles and oil droplets in the micron size range. The emulsion did not settle out with time, attesting to the homogenous nature of the mixture. Tritan® X-100 and Span® 80 were found to produce similar results when mixed in a one-to-one ratio with the product but no further investigations were conducted with these dispersing agents. This technique, using Tween® 80, was found to be compatible with the survival and growth of the test microorganisms and is the basis for this method.

2. Experimental

2.1. Equipment and materials

The test product for this investigation was a prolonged release bovine somatotropin oil formulation. The product consisted of 500 mg of protein suspended in a sesame-oil-based excipient [4]. All procedures prior to sample mixing with Tween® 80 were conducted aseptically in a Class II laminar airflow biohood. All analysts wore powder-free latex gloves and Tyvek sleeves. Controlled temperature incubator rooms at 20–25°C and 30–35°C were used for culture of the microorganisms. Anaerobic jars, anaerobic gas packs, and anaerobic indicators (BBL, Becton Dickinson, Cockeysville, MD) were used for culturing anaerobic streak plates. Disposable 10 μl inoculating loops (Fisher Scientific, Pittsburgh, PA) were used to transfer cultures.

2.2. *Reagents*

Tween® 80 culture bottles were prepared by aliquoting 1.5 ml of Tween® 80 (Sigma Chemical Co., St. Louis, MO) into 4 oz. Boston Round bottles that contained one 1.5 in. stir bar. The

Tween® 80 culture bottles were sterilized by autoclaving at 121°C for 30 min and were stored in the dark at room temperature will all caps tightly closed. The Trypticase Soy Broth (TSB) and Fluid Thioglycollate Medium (FTM) were prepared according to the manufacturer's (BBL) instructions. Both media were dispensed (100 ± 5 ml) into 8 oz. Boston Round bottles before sterilization. TSB was stored at 2–25°C and FTM was stored at 2–8°C after sterilization until use. Not more than the upper 10% of the FTM had a pink color when it was used. Commercially prepared (BBL) TSB and FTM sterile media tubes (20 ml in 20 mm × 148 mm tubes) were used for subculture purposes. Commercially prepared (BBL) Trypticase Soy Agar (TSA) plates (100 mm × 15 mm plates) were used for streak plating and enumeration of microorganism dilutions. Growth promotion testing of the microbial media, as described in USP XXIII, was conducted on all media used in the investigation. TSB is used to recover aerobic microorganisms and FTM is used to recover anaerobic microorganisms.

2.3. Microorganisms

The microorganisms used to validate this methodology are listed below. The microorganisms had been transferred less than five times from the original American Type Culture Collection (ATCC) culture.

(i) *Bacillus subtilis* (ATCC 6633)—gram positive, rod-shaped, aerobic bacterium. Forms endospores.

(ii) *Candida albicans* (ATCC 10231)—gram positive, oval or budding, yeast.

(iii) *Clostridium sporogenes* (ATCC 11437)—gram positive, rod-shaped, anaerobic bacterium. Forms terminal spores.

(iv) *Micrococcus luteus* (ATCC 9341)—gram positive, spherical, aerobic bacterium.

(v) *Pseudomonas aeruginosa* (ATCC 9027)—gram negative, rod-shaped, aerobic bacterium.

All organisms were obtained from the Monsanto Corporate Research, Bioprocess Technology culture collection as frozen stock cultures. After thawing, the cultures were diluted into 0.1% peptamin (Difco, Detroit, MI) to the required concentration for inoculation. No special rejuvenation procedures were used for any test organism. All spore-forming organisms were used as a spore suspension.

2.4. Inoculation of product syringes

The following procedure was used to inoculate Posilac® bovine somatotropin syringes, containing approximately 1.5 g formulated product, with the test microorganisms. An aseptic technique was used. A 1.0 ml sterile syringe and 21 gauge 1.5 in. needle was assembled. About 0.7 ml of the test microorganism dilution containing 10–100 colony forming units (CFU) per 0.1 ml was drawn into the assembled syringe. CFU describes the concentration of viable microorganisms in the suspension and takes into account that two or more microorganisms in suspension may be attached to each other and be observed as one CFU upon assay. The plunger from the product syringe was removed. The product syringe was inoculated with 0.1 ml of the test microorganism dilution. This involved placing the inoculation needle tip close to the center of the product in the syringe. The inoculum was delivered by slowly dragging the needle out of the formulation in the syringe while injecting the test microorganism so that the inoculum remained in the product. A sterile stainless-steel stirring rod (2.4 mm × 150 mm) was placed in the product syringe in such a way as to avoid the needle channel of the syringe. The inoculum was mixed into the product for 10 s by stirring in a clockwise direction for five rotations and then reverse mixing in a counter-clockwise direction for five rotations. This was followed by stirring in a clockwise direction for five rotations with an up and down movement and continuing with the same stirring motion in the counter clockwise direction. The stainless-steel stirring rod was removed from the product syringe in such a way as to avoid removing the product (i.e. rotating the rod against the syringe wall while extracting the stainless-steel rod from the product syringe). The plunger was reinserted about 3–4 mm into the syringe so that it did not expel product.

2.5. Sample preparation

The contents of the inoculated product syringe were dispensed into a screw-capped culture bottle of Tween® 80. One syringe was assayed per culture bottle. The sample was dispensed into the bottom of the bottle to avoid contact with the bottle walls. The bottle was tipped so that the Tween® 80 and product collected in a corner of the bottle. The bottle was gently shaken to mix the Tween® 80 and product. The bottle was then shaken more vigorously so that the stir bar would blend the mixture but no so vigorously that the stir bar left the bottom of the bottle. The bottle was set on a stir plate and stirred so that the stir bar would continue to blend the mixture. Elapsed time from adding the product through blending with a stir bar was 30 s to 1 minute. Aseptically 100 ml of sterile TSB or FTM was added to the product/Tween® 80 mixture with the stir bar spinning. The product/Tween® 80/medium mixture was dispersed into a homogeneous suspension after 1–2 min of rapid stirring on the stir plate. The bottle was then shaken vigorously to completely suspend the mixture if necessary. The bottle walls were carefully inspected to make sure all the product was suspended. Initially the FTM appeared pink indicating that it was oxygenated. The pink color disappeared in <30 min indicating an anaerobic environment. This suspension was incubated at the required temperature (ie. 20–25°C for TSB, 30–35°C for FTM) leaving the bottle caps slightly loose for venting.

2.6. Procedure

The TSB and FTM bottles containing the suspended product were incubated for 14 days. Due to the turbidity of the media bottles they were subcultured on the seventh day. Each TSB and FTM bottle was subcultured by gently swirling the bottle and transferring 10 μl to a sterile 20 ml tube of media using a disposable 10 μl inoculating loop. The TSB tubes were incubated at 20–25°C and the FTM tubes were incubated at 30–35°C. The transfer media were visually examined for growth at least as often as on the third, fourth or fifth day and on the seventh day (last day) of the test period following transfer inoculation. Each initial TSB and FTM culture bottle was streaked on TSA plates on the seventh and 14th days. Each 20 ml TSB and FTM culture tube was streaked on TSA plates on the seventh day. Plates streaked from TSB were incubated aerobically at 20–25°C. Plates streaked from FTM were incubated anaerobically at 30–35°C for 3 days. These plates were used to determine whether growth occurred without visible turbidity and to check the purity of the culture by morphology.

2.7. Bacteriostasis/fungistasis test

The bacteriostatis/fungistasis test was performed using *Bacillus subtilis*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Clostridium sporogenes* as the test microorganisms. Forty product syringes were spiked with a given microorganism at the required concentration (10–100 CFU per syringe). Twenty of the syringes were prepared and incubated with TSB. Twenty of the syringes were prepared and incubated with FTM. Assay of the cultures was as described in Section 2.6.

2.8. Growth rate in the product/Tween® 80 media suspension

A TSB and FTM culture was prepared with each test microorganism in a product/Tween® 80 suspension as described in Section 2.6 using inoculated product syringes. Each bottle was monitored for growth rate and microorganism concentration by determining the number of CFU per milliliter on days 3, 4, 5, 6, and 7 by dilution and spread plating.

2.9. Effect of shaking on FTM

Product was spiked with *Clostridium sporogenes* (9 CFU per syringe) and used to prepare product/Tween® 80 suspensions in FTM as described in Section 2.6. Separate bottles of the culture suspension were stirred, with the caps tightened, for 10 and 60 mins. Following stirring each bottle was hand-shaken vigorously for 1 min. The loss of the pink color is gradual, making it difficult to deter-

mine an endpoint. Therefore each bottle was scored as to whether it lost its pink color at 5 min intervals. A control experiment without the *Clostridium sporogenes* spike was conducted and scored similarly. The culture suspension spiked with *Clostridium sporogenes* and stirred for the various time periods was assayed according to Section 2.6 to determine if this anaerobe could be recovered.

2.10. Effect on Tween® 80 on the survival of the test microorganisms

Individual product syringes spiked with the test microorganisms (10–100 CFU per syringe) were mixed with Tween® 80 as described in Section 2.5. Two bottles of product/Tween® 80 mixture were prepared with each test microorganism. One bottle was held for 10 min at room temperature prior to dilution with media. The second bottle was held for 60 min prior to the addition of medium. The bottles were assayed according to Section 2.6 to determine whether holding the product/Tween® 80 mixture for an extended period of time was detrimental to the recovery of the spiked microorganism.

3. Results and discussion

The Tween® 80 dispersion technique used in this investigation was very effective in producing a homogeneous suspension of the oil-formulated bovine somatotropin particles. The suspension was stable and did not settle out of solution or produce a significant oil layer during the 14 day incubation. The micron size oil droplets and protein particles did result in a very turbid suspension which had a "cream-in-coffee" appearance. Due to the turbidity of the medium suspension, subculture was required in order to visually determine microbial growth. Fig. 1 shows a microscopic view of a product/Tween® 80 FTM suspension inoculated with *Clostridium sporogenes* where oil droplets, amorphous protein particles, and bacteria can be seen. The *Clostridium sporogenes* can range from 1.3–16 μm in length with a diameter of 0.3–1.4 μm [5] with some bacteria having a "tennis racket" appearance due to the refractile nature of the terminal endospore.

Bacteriostasis/fungistasis testing as described in USP XXIII [1] was conducted using the five test microorganisms. Forty product syringes were spiked for each microorganism tested. Of the forty product syringes, half were used to prepare TSB culture suspensions and the other half were used to prepare FTM culture suspensions. Subcultures of the culture suspensions, using a 10 μl disposable inoculating loop into separate media tubes, had growth in all cases, except for *Clostridium sporogenes* which was not recovered in TSB. *Bacillus subtilis*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Candida albicans* were recovered in both the TSB and FTM media. *Clostridium sporogenes* was recovered in FTM media but not TSB media since *Clostridium sporogenes* is an anaerobic organism and TSB is an aerobic medium. Streak plates indicated that all cultures were pure. The growth in the subculture inoculated with the seventh day 10 μl transfer was equivalent to the growth observed in the growth promotion testing. These results demonstrate that the product/Tween® 80 culture suspension is not bacteriostatic/ fungistatic with regard to these microorganisms.

Due to the turbidity of the media containing the product/Tween® 80 mixture, microbial growth in the culture cannot be assessed by visual inspection. Therefore, a subculture step is required in the method. The subculture involves transferring 10 μl of the original product/Tween® 80 culture suspension to a fresh medium tube. Transfer of 10 μl of the culture suspension to a fresh medium tube does not render the tube turbid by visual inspection. In order to validate that 10 μl is a sufficient inoculation volume, growth curves were determined for each of the five test organisms in the product/Tween® 80 culture suspension. In all cases, with the exception of aerobic cultures of the anaerobe *Clostridium sporogenes*, the 10 μl loop was found to transfer more than 100 microorganisms from day 3 and beyond to the subculture media (Table 1). As expected *Clostridium sporogenes* only grew in FTM. The aerobic microorganisms grew in the FTM cultures either because they were not strict aerobes or because sufficient

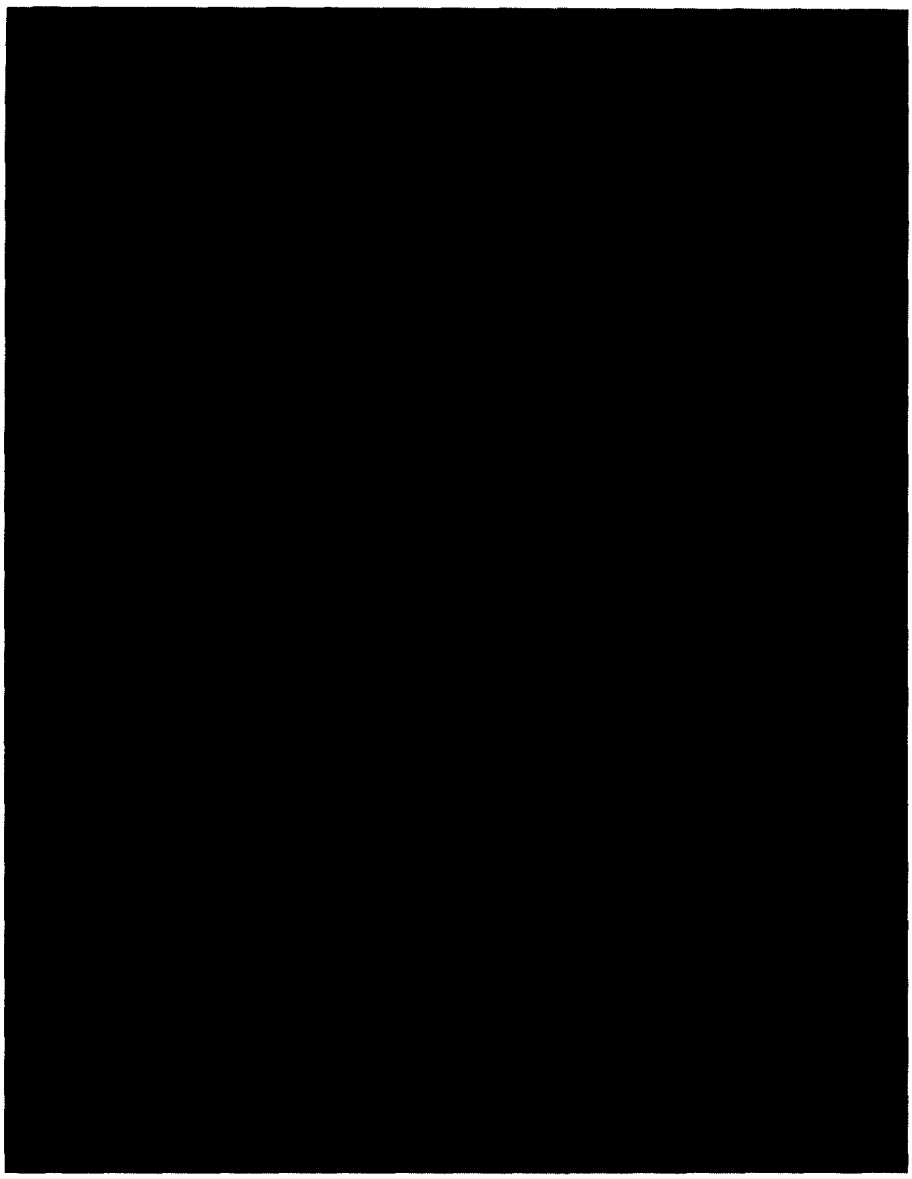

Fig. 1. Microscopic view of a product/Tween® 80 FTM suspension inoculated with *Clostridium sporogenes*. Oil droplets, amorphous protein particles, and bacteria can be seen. Some bacteria containing refractile endospores have a "tennis racket" appearance.

oxygen was present at the liquid/air interface to support their growth. There is potential for not recovering a slow growing microorganism using a 10 μl loop transfer. This concern about slow growing microorganisms also exists for the traditional method of scoring based on observance of visual growth in the product-inoculated culture. These results validate that the 10 μl loop transfer technique will recover the test organisms from the initial culture.

FTM contains chemicals necessary to maintain an anaerobic environment and resazurin, an oxidation/reduction indicator, which gives FTM a pink color if it is oxygenated. If FTM is pink then it might contain sufficient oxygen to prevent the recovery of anaerobic microorganisms. It is typi-

Table 1
Growth rate of microorganisms in culture suspension

Day	Microbial concentration (CFU ml^{-1})									
	B. substilis		*C. albicans*		*M. luteus*		*P. aeruginosa*		*C. sporogenes*	
	TSB	FTM	TSB	FTM	TSB	FTM	TSB	FTM	TSB	FTM
3	1.6×10^7	3.2×10^6	1.3×10^7	1.0×10^6	4.2×10^6	3.3×10^4	6.4×10^8	9.4×10^8	0	5.0×10^7
4	8.3×10^7	8.6×10^6	2.1×10^7	7.9×10^6	2.0×10^6	3.1×10^7	3.3×10^8	3.3×10^8	0	9.0×10^7
5	3.3×10^7	1.2×10^7	3.3×10^7	8.0×10^6	9.3×10^6	6.7×10^7	4.4×10^8	7.8×10^8	0	5.5×10^7
6	5.8×10^7	9.3×10^6	3.3×10^7	8.4×10^6	4.5×10^7	5.3×10^7	2.5×10^8	5.3×10^8	0	5.9×10^7
7	1.5×10^7	9.6×10^6	3.9×10^7	1.3×10^7	7.2×10^7	6.5×10^7	5.9×10^7	6.4×10^8	0	4.9×10^7

cal for FTM to have a slight pink color at its interface with the atmosphere. There was a concern that the shaking necessary to suspend the oil-formulated product in the FTM may introduce enough oxygen to exceed the ability of the FTM to establish and maintain an anaerobic environment. To investigate the effect of stirring and shaking on the FTM culture product syringes were spiked with *Clostridium sporogenes* (average of 9 CFU per syringe) and handled as described in Section 2. Two stirring time points (i.e. 10 or 60 min stirring) were used in triplicate for this investigation. Following stirring each bottle was hand-shaken vigorously for 1 min. The loss of the pink color (i.e. the establishment of an anaerobic environment) was gradual, making it difficult to determine an endpoint. Therefore each bottle was scored every 5 min as to whether it had lost its pink color. When the pink color was gone the time was noted. A control experiment without the *Clostridium sporogenes* spike was also conducted. The culture suspensions spiked with *Clostridium sporogenes* were assayed according to Section 2.6 to determine if this anaerobe was recovered. The loss of pink color occurred within 30 min in all cases. *Clostridium sporogenes* was recovered in all spiked bottles. This indicates that vigorous shaking and mixing did not destroy the ability of FTM to recover anaerobes with similar oxygen sensitivity.

The sterility test procedure requires that the product/Tween® 80 mixture be used to produce a suspension of the product in microbial media. The high Tween® 80 content of the inital 1:1 ratio of product to Tween® 80 may be detrimental to the survival of the test microorganisms. To investigate this concern product was spiked with the test microorganisms and mixed with Tween® 80 as described. Four bottles of product/Tween® 80 mixture without microbial media were prepared from each microorganism tested. Two bottles were held for 10 min at room temperature prior to the addition of dilution media. The other two bottles were held for 60 min prior to the addition of dilution media. TSB was used as the dilution media for all microorganisms, except for *Clostridium sporogenes* where FTM was used. The bottles were assayed as described to determine if holding the product/Tween® 80 mixture for an extended period of time was detrimental to the recovery of the spike microorganism. All microorganisms, except *Pseudomonas aeruginosa*, survived the 60 min incubation in the product/Tween® 80 mixture. *Pseudomonas aeruginosa* survived the 10 min incubation. Therefore, product samples mixed with Tween® 80 should not be held longer than 10 min before dilution with microbial media.

4. Conclusion

The described method for sterility testing of oil-formulated product successfully recovered all the test microrganisms from spiked oil-formulated bovine somatotropin product. All recovered cultures were determined to be pure by streak plat-

ing. The manipulations required to perform the sterility test method were not detrimental to the recovery of the test microorganisms. The growth rate observed for the test microorganisms in the microbial media was rapid and would allow for the microorganisms to be recovered in subculture. Sterility testing is an important quality assessment tool used in sterile drug product quality assurance. Oil-formulated products pose a special sterility testing challenge to the analytical microbiology laboratory because microorganisms need an aqueous environment to grow in. The described sterility test procedure provides a method to meet this goal.

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